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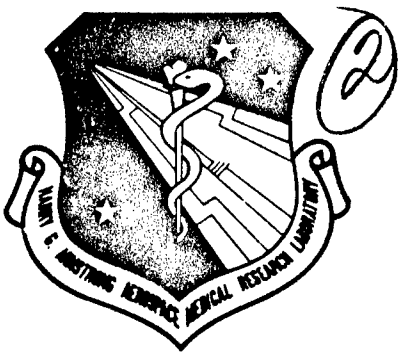

GENOTOXICITY ASSESSMENT OF CHLOROTRIFLUOROETHYLENE TRIMER ACID USING A BATTERY OF *IN VITRO* AND *IN VIVO/IN VITRO* ASSAYS

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AAMRL-TR-90-068

The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

JAMES N. McDOUGAL, Maj, USAF, BSC
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13 ABSTRACT (Maximum 200 words)

Chlorotrifluoroethylene (CTFE) trimer acid, the perhalogenated fatty acid metabolite of the six-carbon oligomer of polychlorotrifluoroethylene (pCTFE), was evaluated in *in vitro* bioassays to assess its potential genotoxic activity. The assays conducted were the Ames *Salmonella*/microsomal mutagenicity assay, the Chinese hamster ovary (CHO) gene mutation assay, the CHO/sister chromatid exchange (SCE) and chromosome aberrations assays, the BALB/c-3T3 cell transformation assay and *in vivo*/*in vitro* unscheduled DNA (UDS) and S phase synthesis assays. CTFE trimer acid did not demonstrate genotoxic potential in any of the *in vitro* assays and was also negative in the UDS assay. However, in the S phase synthesis assay an increase in replicating cells was observed 16 hours after administration of CTFE trimer acid to the study animals by oral gavage. Interestingly, no increase in S phase synthesis was observed at the standard assay time of 48 hours following dosing. This increase in S phase synthesis indicated a rapid response to hepatotoxicity caused by CTFE trimer acid but the results of the test battery would predict no genetic risk from exposure.

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Ames Assay, Chromosomal Aberration, Chlorotrifluoroethylene Trimer Acid, Hepatotoxicity, Mutagenicity, Perhalogenated Fatty Acid, Polychlorotrifluoroethylene, Sister Chromatid Exchange, S-Phase DNA Synthesis, Transformation, Unscheduled DNA Synthesis

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PREFACE

The research reported in this document was conducted by Hazleton Laboratories America, Inc. under a subcontract to NSI Technology Services Corporation in support of the Toxic Hazards Research Unit (THRU). The THRU is the contractor-operated effort of the Toxic Hazards Division of the Harry G. Armstrong Aerospace Medical Research Laboratory located at Wright-Patterson Air Force Base, OH. During the initiation and conduct of these studies Lt Col Michael B. Ballinger and Maj James N. McDougal served consecutively as the contract technical monitor.

The experimental work reported here was begun on 30 October 1989 and completed 5 November 1990. The genotoxicity assays were conducted at the Hazleton Laboratories America facilities in Kensington, MD. The results of their work were reported to NSI in separate reports on each assay. These reports were edited by NSI and organized such that the results of each assay are provided in a separate paper. Each paper is authored by the investigator who conducted the study and a summary (Section 2) is provided to collectively consider the results from the individual studies. The final reports received from Hazleton Laboratories, copies of the raw data, Quality Assurance Statements, and Good Laboratory Practice Compliance and Certification Statements for each of the studies will be archived in the Quality Assurance Archive of the THRU.

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ABBREVIATIONS

AF	Acentric fragment
BrdU	5-Bromo-2'deoxyuridine
°C	Degrees Centigrade
CE	Cloning efficiency
CHO	Chinese hamster ovary
Ci	Curies
cm	Centimeter
CoA	Coenzyme A
CP	Cyclophosphamide
CTFE	Chlorotrifluoroethylene
DMN	Dimethylnitrosamine
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EMEM	Eagles's minimum essential medium
F-344	Fischer 344
FBS	Fetal bovine serum
FPG	Fluorescence-plus-Giemsa
g	Gram
h	Hour
HGPRT	Hypoxanthine-guanine phosphoribosyl transferase
^{3}HTdr	Tritiated thymidine
ip	Intraperitoneal
kg	Kilogram
M	Molar
MCA	3-Methylcholanthrene
µCi	Microcurie
µg	Microgram
µL	Microliter
µM	Micromolar
mg	Milligram
min	Minute
mL	Milliliter
mm	Millimeter
mM	Millimolar
MMC	Mitomycin C
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NNG	Net nuclear grains
NTP	National Toxcicology Program
p	Probability
PBS	Phosphate buffered saline
%	Percent
pCTFE	Polychlorotrifluoroethylene
RI	Replicative index
rpm	Revolutions per minute
SCE	Sister chromatid exchange
SD	Standard deviation
SE	Standard error
TG	6-Thioguanine
THRU	Toxic Hazards Research Unit
U	Units
UDS	Unscheduled DNA synthesis
UV	Ultraviolet
v/v	Volume per volume

VC	Vehicle control
w/v	Weight per volume
xg	Forces of gravity

SECTION 1

INTRODUCTION

C. S. Godin

Polychlorotrifluoroethylene (pCTFE), a candidate hydraulic fluid used in Department of Defense advanced weapon systems, is a perhalogenated mixture of primarily 6- and 8-carbon oligomers of chlorotrifluoroethylene (CTFE) that are referred to as CTFE trimer and CTFE tetramer, respectively.

Subchronic inhalation exposure of pCTFE to rats caused an increase in both relative liver weight and hepatocytic peroxisomes (Kinkead et al., 1990). The administration of pCTFE by oral gavage to rats for 14 days resulted in similar effects as well as an increase in the rate of peroxisomal ß-oxidation of palmitoyl coenzyme A (CoA)(DelRaso et al., submitted). The administration of CTFE trimer by oral gavage for 14 days in the same study caused a significant increase in relative liver weight but did not cause a significant increase in ß-oxidation; CTFE tetramer produced a significant increase in both parameters. In another study, the fatty acid metabolite of CTFE trimer, CTFE trimer acid, was administered weekly by oral gavage for 3,6,9, or 12 months and caused a significant increase in ß-oxidation at the three month timepoint only but no significant increase in relative liver weight; concurrent administration of CTFE tetramer acid to separate groups of animals caused a significant increase in both parameters that was manifested after three months of weekly dosing (Kinkead et al., 1991).

Many compounds cause an increase in hepatocytic peroxisomes and many are structural analogs of the hypolipidemic agent, clofibrate (Lalwani et al., 1983). However, numerous industrial chemicals such as phthalate ester plasticizers and phenoxy acid herbicides (Reddy et al., 1976; Kawashima et al., 1984) can also induce this effect. Recently perfluoro-*n*-decanoic acid, a perfluorinated fatty acid, was shown to cause hepatomegaly, peroxisomal proliferation, and an increase in the activity of the peroxisomal enzyme, fatty acyl-CoA oxidase (Olson et al., 1982; Van Rafelghem, 1985; Harrison et al., 1988). Therefore, perhalogenated fatty acids represent a new class of peroxisome proliferators.

Peroxisome proliferation has been correlated with hepatocarcinogenic potency (Reddy et al., 1980; Elcombe, 1985) and CTFE trimer acid has been shown to act as a tumor promoter (Godin et al., 1990). It has been suggested that peroxisome proliferators promote tumor formation by inducing oxidative stress (Reddy and Lalwani, 1983; Reddy and Rao, 1987; Rao and Reddy, 1987) or by providing a mitogenic stimulus (NTP, 1982; Ciriolo et al., 1982; Ward et al., 1984; Marsman et al., 1988). A mitogenic stimulus causes an increase in the amount of S-phase DNA synthesis and ultimately results in hepatomegaly. Oral administration of pCTFE has resulted in a ~2-fold increase in the amount of DNA per gram of liver (Godin, unpublished observation), and hepatomegaly has been reported following inhalation exposures and oral dosing with pCTFE oligomers and CTFE tetramer acid as previously discussed; CTFE trimer acid has not been shown to cause hepatomegaly.

While peroxisome proliferation has been correlated with hepatocarcinogenic potency peroxisome proliferators as a class have not been shown to be genotoxic (Warren et al., 1980; Gupta et al., 1985). Because CTFE trimer acid is a member of this new class of peroxisome proliferators, and to more fully evaluate the potential human health hazards of these compounds, *in vitro* and *in vivo* tests were conducted to determine their genotoxic potential.

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SECTION 2

CTFE TRIMER ACID GENOTOXICITY SUMMARY EVALUATION

B. Myhr

The compound, CTFE trimer acid, was tested for potential genotoxic activity by application to several *in vitro* assays, as described below, and to a sensitive *in vivo* assay for DNA damage and cytotoxicity in the liver. The substance was a clear, colorless, slightly viscous liquid that was found to be soluble in dimethylsulfoxide (DMSO) at concentrations at least as high as 500 mg/mL. Solutions were prepared in DMSO just prior to use in each *in vitro* assay. Glass containers and pipets were used, and the treatments of mammalian cells *in vitro* were conducted in glass flasks. After dilution of the stock solutions into culture medium, complete solubility was maintained to at least 1670 μg/mL; precipitation was noted at 5020 μg/mL. For the *in vivo* study, CTFE trimer acid was solubilized in corn oil and administered by oral gavage.

The Ames *Salmonella* reverse mutation assay was performed by the preincubation method in order to maximize the interaction between the bacteria and CTFE trimer acid. Strains TA98, TA100, TA1535, TA1537, and TA1538 were used in the presence and absence of a rat liver S9 metabolic activation system (Aroclor 1254-induced). CTFE trimer acid became slightly toxic at a concentration of 3330 μg/plate, with or without S9, yet the toxicity did not become excessive at the maximum applied concentration of 10,000 μg/plate. A dose range of 250 μg/plate to 10,000 μg/plate was assayed for the induction of revertants. No increases in revertants were obtained, so CTFE trimer acid showed no detectable mutagenic activity in this bacterial system.

In mammalian cell culture, CTFE trimer acid was tested for mutagenic activity at the HGPRT locus in CHO cells. After a 4-h exposure period, significant toxicity was obtained at the highest applied dose of 1000 μg/mL; with addition of S9, however, the 1000 μg/mL treatment was lethal. The mutation assay performed without S9 over a concentration range of 50 to 1000 μg/mL yielded a survival range of 55 to 123% and no significant increase in mutant frequency. With S9, the high dose was reduced to 800 μg/mL, which yielded 26% survival; again, no increase in mutant frequency was observed. Therefore, CTFE trimer acid was toxic to cultured CHO cells but did not induce any detectable mutagenesis at the HGPRT locus.

CHO cells were also examined for cytogenetic damage in assays for chromosomal aberrations and sister chromatid exchanges (SCE) after treatment with CTFE trimer acid. In the absence of S9, CTFE trimer acid was lethal at a concentration of 1670 μg/mL and caused substantial cell cycle delay (indicating toxicity) at 167 and 502 μg/mL. Accordingly, a dose range of 5 to 167 μg/mL was analyzed for SCE. No significant increase was observed, and too few metaphase cells were available at the next highest dose of 502 μg/mL to allow an analysis. A delayed harvest time of 20 h was used for the chromosome aberrations assay in order to compensate for the toxicity. Over the assayed dose range of 254 to 1010 μg/mL, no induction of chromosomal aberration was observed. With 2-h treatments in the presence of S9, lethality was observed at a concentration of 5020 μg/mL, and only slight cell cycle delay occurred at 1670 μg/mL. No significant increase in SCE was obtained for an assayed dose range of 50 to 1670 μg/mL. A normal harvest time of 10 h was used for the chromosome aberrations assay over a dose range of 374 to 2490 μg/mL. No increase in aberrations was observed, except for the 2490 μg/mL treatment, which was excessively toxic. Due to this toxicity and the absence of any

dose-related effect, CTFE trimer acid was evaluated as nonclastogenic with S9. Thus, cytogenetic damage was not induced by CTFE trimer acid in cultured CHO cells in the absence or presence of S9.

An *in vivo* assessment of genetic activity was performed by dosing male F-344 rats with four doses of CTFE trimer acid ranging from 24.8 mg/kg to 198 mg/kg. After single oral administrations, primary hepatocyte cultures were established to determine the degree of DNA repair (unscheduled DNA synthesis, UDS) and DNA replication (S-phase) by labeling with ^{3}H-thymidine, followed by autoradiographic analysis. The cultures were prepared approximately 16 h after treatment of the animals for the UDS analysis and 47-48 h after treatment of additional animals for S-phase analysis. None of the treatments caused any increase in nuclear labeling or the percent of cells in DNA repair relative to the control rats. Therefore, CTFE trimer acid did not induce any detectable UDS. However, evidence for liver toxicity was obtained from the S-phase analysis. At the standard assay time of approximately 48 h, no increase in S-phase cells was observed, but the 16-h cultures prepared for UDS analysis did show S-phase induction. Small, but significant, dose-related increases occurred for all treatments except for the low dose of 24.8 mg/kg. At a dose of 198 mg/kg, an average of 2.2% of the cells were in S-phase, compared to an average value of 0.2% in the control rats. This increase in hepatocyte replication indicated a rapid response to hepatotoxicity caused by the CTFE trimer acid treatments.

CTFE trimer acid was assayed for its ability to induce morphological transformation *in vitro* in cultures of mouse BALB/c-3T3 cells, both in the presence and absence of a rat liver S9 metabolic activation system. The treatment periods were 2 h with S9 and 72 h without S9, and two independent trials were conducted under each test condition. The treatments in the presence of S9 resulted in less toxicity, as measured by the clonal survivals of ouabain-resistant cells in the presence of the wildtype monolayer cultures. Without S9, no response was obtained for a dose range of 100 to 700 μg/mL, which yielded a survival range of 47 to 95%. The dose range was adjusted to 200 to 800 μg/mL for the repeat trial, which yielded a survival range of 11 to 93%. This trial was also evaluated as negative. With addition of S9, both trials were conducted over a dose range of 500 to 2000 μg/mL, which caused survivals ranging from 6 to 108%. Dose-related responses were not obtained, and both trials were evaluated as negative. Therefore, no evidence was obtained for transforming activity *in vitro* by CTFE trimer acid.

The results of the above genetic tests indicated that CTFE trimer acid does not interact with genetic material. No responses were obtained for any of the *in vitro* genetic endpoints tested, using CTFE trimer acid treatments over a wide toxicity range. Furthermore, no response was observed *in vivo* from the rat liver UDS study. The rat liver study did indicate the rapid onset of a low-level hepatotoxicity. Thus, the results of the genetic test battery would predict no genetic risk from exposures to CTFE trimer acid, but slight physiological stress to the liver could occur upon ingestion of the compound.

SECTION 3

MUTAGENICITY TEST ON CTFE TRIMER ACID IN THE *SALMONELLA*/REVERSE MUTATION ASSAY (AMES TEST) PREINCUBATION METHOD

T.E. Lawlor

INTRODUCTION

The mutagenic activity of chlorotrifluoroethylene (CTFE) trimer acid was examined in the *Salmonella*/Reverse Mutation Assay (Ames Test), Preincubation Method. This assay evaluated the test article and/or its metabolites for their ability to induce reverse mutations at the histidine locus in the genome of specific *Salmonella typhimurium* tester strains both in the presence and absence of an exogenous metabolic activation system of mammalian microsomal enzymes derived from Aroclor-induced rat liver.

The Ames Test detects both frameshift and/or base pair substitution point mutations in bacteria and has been shown to be a sensitive, rapid, and accurate indicator of the mutagenic activity of many materials including a wide range of chemical classes. Both base pair substitution mutations and frameshift mutations can be detected by utilizing several different tester strains. Tester strains TA98, TA1537, and TA1538 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. TA1535 is reverted by base substitution mutagens and TA100 is reverted by mutagens which cause both frameshifts and base substitutions.

The strains of *Salmonella typhimurium* used in this assay were all histidine auxotrophs by virtue of conditionally lethal mutations in their histidine operon. When these histidine-dependent cells (*his*-) were exposed to the test article and grown under selective conditions (the use of minimal media with a trace amount of histidine) only those cells that reverted to histidine independence (*his*+) were able to form colonies. The trace amount of histidine in the media permitted the plated bacteria to undergo a few cell divisions that are essential for the full expression of mutagenesis. The *his*+ revertants were readily discernible as colonies against the limited background growth of the *his*- cells.

MATERIALS AND METHODS

The experimental materials, methods, and procedures were based on those described by Ames et al. (1975) and Yahagi et al. (1975).

Media and Reagents

Top Agar for Selection of Histidine Revertants: Minimal top agar was prepared with 0.7% agar (w/v) and 0.5% NaCl (w/v). The agar was sterilized by autoclaving, distributed into sterile bottles, and stored at room temperature prior to use. Immediately before use in the mutagenicity assay, the top agar was melted and supplemented with a sterile solution containing 0.5 mM L-histidine and 0.5 mM D-biotin (10% v/v).

Minimal Bottom Agar: The bottom agar was Vogel-Bonner minimal medium E (Vogel and Bonner, 1956), supplemented with 0.2% (w/v) glucose.

Nutrient Broth: The nutrient broth used for growing overnight cultures of the tester strains was Vogel-Bonner salt solution supplemented with 2.5% (w/v) Oxoid Nutrient Broth No. 2 (dry powder).

Exogenous Metabolic Activation

Liver Microsomal Enzymes - S9 Homogenate: The S9 liver homogenate was prepared from male Sprague-Dawley rats that had been injected intraperitoneally (ip) with Aroclor 1254 (500 mg/kg) five days prior to preparation of the homogenate and was purchased from Molecular Toxicology, Inc. The S9 homogenate was characterized for its ability to metabolize selected promutagens to their mutagenic forms in the Ames Test as described by deSerres and Shelby (1979).

S9 Reaction Mixture: A mixture of S9 homogenate and cofactors was prepared immediately before use in those assays that required metabolic activation. One mL of this reaction mixture contained the following components:

H_2O	0.70 mL
1.00M NaH_2PO_4/Na_2HPO_4, pH 7.4	0.10 mL
0.25M Glucose-6-phosphate	0.02 mL
0.10M NADP	0.04 mL
0.2M $MgCl_2$/0.825M KCl	0.04 mL
S9 Homogenate	0.10 mL

Test System

Tester Strains: The tester strains employed were the *Salmonella typhimurium* histidine auxotrophs TA98, TA100, TA1535, TA1537, and TA1538. Frozen permanent stocks of each tester strain were prepared by growing fresh overnight cultures, adding DMSO (0.09mL/mL of culture), and freezing 1.5 mL aliquots at ≤ -70°C. The description of the tester strains that follows is based on that provided by Ames et al. (1975).

In addition to a mutation in the histidine operon discussed in the introduction, all strains contained two additional mutations that enhanced their sensitivity to some mutagenic compounds. The *rfa* wall mutation results in the loss of one of the enzymes responsible for the synthesis of part of the lipopolysaccharide barrier of the bacterial cell wall. The resulting cell wall deficiency increases permeability to certain classes of chemicals such as those containing large ring systems (i.e. benzo(a)pyrene) that would otherwise be excluded by a normal intact cell wall. The second mutation, a deletion of the *uvrB* gene, results in a deficient DNA excision repair system that greatly enhances the sensitivity of these strains to some mutagens. Because the *uvrB* deletion extends through the *bio* gene, all strains containing this deletion also required biotin for growth.

Strains TA98 and TA100 also contained the R-factor plasmid, pKM101, that further increases the sensitivity of these strains to some mutagens. The mechanism by which this plasmid increases sensitivity to mutagens has been suggested to be by modification of an existing bacterial DNA repair polymerase complex involved with the mismatch-repair process.

Master Plates: Master plates of the tester strains were prepared by streaking each strain from a frozen permanent stock onto minimal agar appropriately supplemented with histidine (260 μM), biotin (2 μM), and for strains containing the R-factor, ampicillin (25 μg/mL). Tester strain master plates were stored at 6 ± 4°C.

Preparation of Overnight Cultures: Overnight cultures were prepared by transferring a colony from the appropriate master plate to a flask containing culture medium. In order to assure that cultures were harvested in late log phase, the length of incubation was determined by spectrophotometric

monitoring. Inoculated flasks were placed in a shaker/incubator that was programmed to begin operation (shaking, 125 ± 25 rpm; incubation, 37 ± 2°C) so that the overnight cultures were in log phase or late log phase when turbidity monitoring began. Cultures were harvested once a predetermined turbidity was reached because overgrowth of cultures can result in losses of sensitivity to some mutagens.

Confirmation of Tester Strain Genotypes: Tester strain cultures were checked for the following genetic markers on the day of their use in the mutagenicity assay:

rfa Wall Mutation: The presence of the *rfa* wall mutation was confirmed by demonstration of sensitivity to crystal violet. An aliquot of an overnight culture of each strain was overlaid onto plates containing selective media and an antibiotic sensitivity disk containing 10 μg of crystal violet was added. Sensitivity was demonstrated by inhibition of bacterial growth in a zone immediately surrounding the disk.

pKM101 Plasmid R-factor: The presence of the pKM101 plasmid was confirmed for tester strains TA98 and TA100 by demonstration of resistance to ampicillin. An aliquot of an overnight culture of each strain was overlaid onto plates containing selective media and an antibiotic sensitivity disk containing 10 μg of ampicillin was added. Resistance was demonstrated by bacterial growth in the zone immediately surrounding the disk.

Characteristic Number of Spontaneous Revertants: The mean number of spontaneous revertants per plate in the vehicle controls that are characteristic of the respective strains was demonstrated by plating 100 μL aliquots of the culture along with the appropriate vehicle on selective media.

Experimental Design

Test Article Characteristics and Handling: The CTFE trimer acid (ID# 10-86-63, IR# 14240) was provided by the U.S. Air Force. The clear, colorless, viscous liquid (BP 82-85°C/10^3 mmHg) was stored at room temperature in its original container. DMSO was used as the vehicle and the test article formed a solution at 200 mg/mL which was the most concentrated stock dilution of test article prepared. The test article remained in solution at all subsequent dilutions prepared for the mutagenicity assay.

Dose Rangefinding Study: The dose rangefinding study was conducted using tester strain TA100 both in the presence and absence of S9 microsomal enzymes. TA100 was used because the growth inhibitory effect (cytotoxicity) of the test article on this strain is generally representative of the effect observed on the other tester strains. In addition, TA100's comparatively high number of spontaneous revertants per plate affords the ability to discern gradations of cytotoxicity from routine experimental variation. Also, the cytotoxicity induced by a test article in the presence of microsomal enzymes may vary greatly from that observed in the absence of microsomal enzymes. Therefore, this would require that different test article dose ranges be tested in the mutagenicity assay based on the presence or absence of the microsomal enzymes.

Ten doses of test article ranging from 10 to 10,000 μg per plate were tested. The assay was conducted using one plate per dose in the presence and absence of S9 microsomal enzymes. Cytotoxicity in this study was detectable

as a decrease in the number of revertant colonies per plate and/or a thinning or disappearance of the bacterial background lawn. Routinely, the maximum dose selected to be tested in the mutagenicity assay should demonstrate cytotoxicity.

Mutagenicity Assay: The mutagenicity assay was performed using tester strains TA98, TA100, TA1535, TA1537, and TA1538 and used three plates per dose in the presence and absence of S9 microsomal enzymes. Six doses of the test article ranging from 250 to 10,000 μg per plate were tested along with the appropriate vehicle and positive controls. The doses tested were selected based on the results of the dose rangefinding study and reflect the fact that the exposure of the test system to the test article does not cease at the end of the 20-min preincubation period. A dose of 10,000 μg per plate indicates that the bacteria are exposed to a concentration of 15,400 μg of test article/mL of preincubation mixture for 20 min prior to being combined with 2 mL of overlay agar and being overlaid onto 25 mL of bottom agar.

Frequency and Route of Administration: The test system was exposed to the test article via the preincubation modification of the Ames Test originally described by Yahagi et al. (1975). This methodology has been shown to detect mutagenicity with certain classes of chemicals, such as nitrosamines or volatile compounds, that may not be detected in the standard plate incorporation method. All doses of test article, vehicle controls, and positive controls were preincubated and plated in triplicate.

Positive Controls: The following compounds were used as the positive controls: 2-aminoanthracene (Sigma Chemical Co., practical grade); 2-nitrofluorene (Aldrich Chemical Co., 98%); sodium azide (Sigma Chemical Co., practical grade; ICR-191 (Polysciences Inc., > 95% pure). All combinations of positive controls and tester strains plated concurrently with the assay are listed below.

POSITIVE CONTROL AND TESTER STRAIN COMBINATIONS

Tester Strain	S9 Mix	Positive Control	Conc. per Plate
TA98	+	2-aminoanthracene	2.5 μg
TA98	-	2-nitrofluorene	1.0 μg
TA100	+	2-aminoanthracene	2.5 μg
TA100	-	sodium azide	2.0 μg
TA1535	+	2-aminoanthracene	2.5 μg
TA1535	-	sodium azide	2.0 μg
TA1537	+	2-aminoanthracene	2.5 μg
TA1537	-	ICR-191	2.0 μg
TA1538	+	2-aminoanthracene	2.5 μg
TA1538	-	2-nitrofluorene	1.0 μg

Vehicle Controls: Appropriate vehicle controls were plated for all tester strains both in the presence and absence of S9 microsomal enzymes. The vehicle control was plated, using an aliquot of vehicle equal to the aliquot of test article dilution plated, along with an aliquot of the appropriate tester strain, on selective agar.

Sterility Controls: In order to determine the sterility of the test article, the highest test article dose tested in the mutagenicity assay was checked for sterility by plating an aliquot volume equal to that used in the assay on selective agar. In order to determine the sterility of the S9 mixture and the 0.1 M phosphate buffer, a 0.5 mL aliquot of each was plated on selective agar.

Plating Procedures: The plating procedures employed were similar to those described by Ames et al. (1975) and Yahagi et al. (1975). These procedures were employed for both the dose rangefinding study and the mutagenicity assay. Each plate was labeled with a code system that identified the test article, tester strain, test phase, dose, and activation condition.

The test article was diluted and the S9 mixture was prepared immediately before their use in any experimental procedure. When S9 mix was required, 0.5 mL of S9 mix was added to 13 x 100 mm glass culture tubes pre-heated to 37 ± 2°C. To these tubes were added 100 µL of appropriate tester strain and 50 µL of vehicle or test article dilution. When S9 mix was not required, 0.5 mL of 0.1M phosphate buffer was substituted for the S9 mixture. After vortexing, the mixture was allowed to incubate for 20 ± 2 min at 37 ± 2°C. Two mL of molten selective top agar were added to each tube and the mixture was vortexed and overlaid onto the surface of 25 mL of minimal bottom agar contained in a 15 x 100 mm petri dish. After the overlay had solidified, the plates were inverted and incubated for 48 ± 8 h at 37 ± 2°C.

Scoring Plates: Plates that were not scored immediately after the 48-h incubation period were held at 6 ± 4°C until scoring could be completed.

The condition of the background bacterial lawn was evaluated for evidence of cytotoxicity caused by the test article by using a dissecting microscope. The cytotoxicity was scored relative to the vehicle control plate and has been noted along with the revertant counts for all plates at that dose on the data tables using the code system presented in Appendix 3-A. In addition to the cytotoxicity, any test article precipitate observed on the plates was also noted at the appropriate dose on the data tables, again using the code system presented in Appendix 3-A.

Revertant colonies for a given tester strain and activation condition were counted either entirely by automated colony counter or entirely by hand. If the plates contained sufficient test article precipitate to interfere with automated colony counting, then they were counted manually.

For all replicate platings, the mean number of revertants per plate was calculated and the standard deviation around the mean was also calculated.

RESULTS AND DISCUSSION

Dose Rangefinding Study

The doses of CTFE trimer acid for testing in the mutagenicity assay were selected based on the results of the dose rangefinding study employing tester strain TA100. Ten doses of test article ranging from 10 to 10,000 µg per plate were tested and the results are presented in Table 3-1. Cytotoxicity was observed at a dose of 3330 µg per plate and higher both in the presence and absence of S9 microsomal enzymes as evidenced by the reduced number of revertants per plate and/or the thinning of the bacterial background lawn.

TABLE 3-1. DOSE RANGEFINDING STUDY FOR CTFE TRIMER ACID

	TA100 Revertants per plate			
	With S9		Without S9	
µg\Plate	Number of Colonies/Plate	Appearance of Background Lawn*	Number of Colonies/Plate	Appearance of Background Lawn*
0.00 (Vehicle) (50 µL)	92	1	93	1
CTFE Trimer Acid				
10.0	124	1	74	1
33.3	100	1	67	1
66.7	95	1	97	1
100	106	1	77	1
333	113	1	78	1
667	119	1	98	1
1000	127	1	93	1
3330	100	2	92	2
6670	88	2	41	3
10000	59	3	44	3

* Background Lawn Evaluation Codes:

1 = normal
2 = slightly reduced
3 = moderately reduced
sp = slight precipitate
mp = moderate precipitate (requires hand count)
4 = extremely reduced
5 = absent
6 = obscured by precipitate
hp = heavy precipitate (requires hand count)

Mutagenicity Assay

The results of the dose rangefinding study were used to select 6 doses to be tested in the mutagenicity assay. The doses selected for the mutation assay ranged from 250 to 10,000 µg per plate. The mutagenicity assay results for CTFE trimer acid are presented in Tables 3-2 and 3-3. All data were acceptable and no positive increase in the number of histidine revertants per plate was observed either in the presence or absence of S9 microsomal enzymes. All criteria for a valid study were met (See Appendix 3-B).

CONCLUSION

The results of the *Salmonella*/Reverse Mutation assay (Ames Test), Preincubation Method, indicated that under the conditions of this study, CTFE trimer acid did not cause a positive increase in the number of histidine revertants per plate for any of the tester strains either in the presence or absence of S9 microsomal enzymes prepared from Aroclor-induced rat liver.

TABLE 3-2. INDIVIDUAL PLATE COUNTS FROM AMES ASSAY OF CTFE TRIMER ACID

		Revertants per Plate															Background
	Dose/Plate	TA98			TA100			TA1535			TA1537			TA1538			Lawn*
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	
MICROSOMES: Rat Liver																	
VEHICLE CONTROL		41	33	30	97	62	111	16	9	13	10	9	7	13	13	27	1
TEST ARTICLE	250 µg	31	33	31	99	101	86	15	12	9	7	5	8	17	23	21	1
	500 µg	25	45	40	121	93	92	5	12	6	11	8	3	23	19	20	1
	1000 µg	41	25	27	91	94	89	17	15	11	14	11	7	24	19	17	1
	2500 µg	26	19	29	101	105	105	11	12	12	7	7	8	26	27	17	1
	5000 µg	25	26	25	102	84	91	12	11	5	4	9	7	18	19	10	2
	10000 µg	27	18	21	85	70	89	9	12	8	3	3	5	14	13	14	2
POSITIVE CONTROL **		1172	1136	1146	1032	1202	1043	155	142	116	205	193	244	1347	1226	1309	1
MICROSOMES: None																	
VEHICLE CONTROL		14	24	20	103	103	108	19	12	15	6	4	3	21	15	27	1
TEST ARTICLE	250 µg	19	18	24	86	93	91	16	10	17	10	4	6	18	15	12	1
	500 µg	18	22	26	80	86	102	15	13	10	4	7	10	16	19	20	1
	1000 µg	20	25	26	104	67	90	12	16	16	7	6	6	12	7	20	1
	2500 µg	17	23	20	78	68	91	14	16	13	6	8	11	15	20	17	2
	5000 µg	22	14	12	68	86	65	14	12	9	3	3	8	13	14	15	2
	10000 µg	13	15	14	56	47	65	11	11	4	2	4	2	11	18	9	3
POSITIVE CONTROL ***		200	193	186	662	734	570	467	545	519	1753	1779	1653	367	422	410	1

* Background Lawn Evaluation Codes:

1 = normal; 2 = slightly reduced; 3 = moderately reduced
4 = extremely reduced; 5 = absent; 6 = obscured by precipitate
sp = slight precipitate; mp = moderate precipitate (requires hand count); hp = heavy precipitate (requires hand count)

** TA98 2-aminoanthracene 2.5 µg/plate
TA100 2-aminoanthracene 2.5 µg/plate
TA1535 2-aminoanthracene 2.5 µg/plate
TA1537 2-aminoanthracene 2.5 µg/plate
TA1538 2-aminoanthracene 2.5 µg/plate

*** TA98 2-nitrofluorene 1.0 µg/plate
TA100 sodium azide 2.0 µg/plate
TA1535 sodium azide 2.0 µg/plate
TA1537 ICR-191 2.0 µg/plate
TA1538 2-nitrofluorene 1.0 µg/plate

TABLE 3-3. SUMMARY OF TEST RESULTS FOR AMES ASSAY OF CTFE TRIMER ACID

		Mean Revertants per Plate with Standard Deviations										
	Dose/Plate	TA98		TA100		TA1535		TA1537		TA1538		Background
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Lawn*
MICROSOMES: Rat Liver												
VEHICLE CONTROL		35	6	90	25	13	4	9	2	18	8	1
TEST ARTICLE	250 µg	32	1	95	8	12	3	7	2	20	3	1
	500 µg	37	10	102	16	8	4	7	4	21	2	1
	1000 µg	31	9	91	3	14	3	11	4	20	4	1
	2500 µg	25	5	104	2	12	1	7	1	23	6	1
	5000 µg	25	1	92	9	9	4	7	3	16	5	2
	10000 µg	22	5	81	10	10	2	4	1	14	1	2
POSITIVE CONTROL **		1151	19	1092	95	138	20	214	27	1294	62	1
MICROSOMES: None												
VEHICLE CONTROL		19	5	105	3	15	4	4	2	21	6	1
TEST ARTICLE	250 µg	20	3	90	4	14	4	7	3	15	3	1
	500 µg	22	4	89	11	13	3	7	3	18	2	1
	1000 µg	24	3	87	19	15	2	6	1	13	7	1
	2500 µg	20	3	79	12	14	2	8	3	17	3	2
	5000 µg	16	5	73	11	12	3	5	3	14	1	2
	10000 µg	14	1	56	9	9	4	3	1	13	5	3
POSITIVE CONTROL ***		193	7	655	82	510	40	1728	67	400	29	1

* Background Lawn Evaluation Codes:
1 = normal; 2 = slightly reduced; 3 = moderately reduced
4 = extremely reduced; 5 = absent; 6 = obscured by precipitate
sp = slight precipitate; mp = moderate precipitate (requires hand count); hp = heavy precipitate (requires hand count)

** TA98 2-aminoanthracene 2.5 µg/plate
TA100 2-aminoanthracene 2.5 µg/plate
TA1535 2-aminoanthracene 2.5 µg/plate
TA1537 2-aminoanthracene 2.5 µg/plate
TA1538 2-aminoanthracene 2.5 µg/plate

*** TA98 2-nitrofluorene 1.0 µg/plate
TA100 sodium azide 2.0 µg/plate
TA1535 sodium azide 2.0 µg/plate
TA1537 ICR-191 2.0 µg/plate
TA1538 2-nitrofluorene 1.0 µg/plate

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APPENDIX 3-A

BACTERIAL BACKGROUND LAWN EVALUATION CODE

The condition of the background bacterial lawn is evaluated both macroscopically and microscopically (using a dissecting microscope) for indications of cytotoxicity and test article precipitate as follows:

CODE	DEFINITION	CHARACTERISTICS OF BACKGROUND LAWN
1	Normal	A healthy microcolony lawn.
2	Slightly Reduced	A noticeable thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
3	Moderately Reduced	A marked thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
4	Extremely Reduced	An extreme thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
5	Absent	A complete lack of any microcolony lawn.
6	Obscured by Precipitate	The background bacterial lawn cannot be accurately evaluated due to microscopic and/or macroscopic test article precipitate.

Evidence of macroscopic test article precipitate on the plates is recorded by addition of the following precipitate code to the code number used to evaluate the condition of the background bacterial lawn.

SP	Slight Precipitate	Noticeable macroscopic precipitate on the plate, however, the precipitate does not influence automated counting of the plate.
MP	Moderate Precipitate	The amount of macroscopic precipitate on the plate would interfere with automated counting, thus, requiring the plate to be hand counted.
HP	Heavy Precipitate	The large amount of macroscopic precipitate on the plate makes the required hand counting difficult.

Example: 4-MP would indicate a plate observed to have an extremely reduced background lawn which had to be counted manually due to the marked amount of macroscopic test article precipitate.

APPENDIX 3-B

CRITERIA FOR THE DETERMINATION OF A VALID TEST

The following criteria must be met for the assay to be considered valid:

rfa Wall Mutation: In order to demonstrate the presence of the deep rough mutation, all tester strain cultures must exhibit sensitivity to crystal violet.

pKM101 Plasmid R-Factor: In order to demonstrate the presence of the pKM101 Plasmid R-factor, all tester strains must exhibit resistance to ampicillin.

Characteristic Number of Spontaneous Revertants: All tester strain cultures must exhibit a characteristic number of spontaneous revertants per plate in the vehicle controls. The acceptable ranges are as follows:

TA98	8 - 60
TA100	60 - 240
TA1535	4 - 45
TA1537	2 - 25
TA1538	3 - 35

Tester Strain Culture Density: In order to ensure that appropriate numbers of bacteria are plated, tester strain culture density must be greater than or equal to 5.0×10^8 bacteria per mL and/or have reached a target level of turbidity demonstrated to produce cultures with a density greater than or equal to 5.0×10^8.

Positive Control Values: All positive controls must exhibit at least a 3-fold increase in the number of revertants per plate over the mean value for the vehicle control for the respective strain.

Cytotoxicity: A minimum of three non-toxic doses are required to evaluate assay data.

Evaluation of Test Results

Tester Strains TA98 and TA100: For a test article to be considered positive, it must cause at least a 2-fold increase in the mean revertants per plate of at least one tester strain over the mean vehicle control value for that tester strain. This increase in the mean number of revertants per plate must be accompanied by a dose response to increasing concentrations of the test article.

Tester Strains TA1535, TA1537, and TA1538: For a test article to be considered positive, it must cause at least a 3-fold increase in the mean revertants per plate of at least one tester strain over the mean vehicle control value for that tester strain. This increase in the mean number of revertants per plate must be accompanied by a dose response to increasing concentrations of the test article.

SECTION 4

MUTAGENICITY TEST ON CTFE TRIMER ACID IN THE CHO/HGPRT FORWARD MUTATION ASSAY

R.R. Young

ABSTRACT

The objective of this assay was to evaluate the ability of CTFE trimer acid to induce forward mutations at the HGPRT locus in CHO cells both with and without metabolic activation by S9 microsomal enzymes.

CTFE trimer acid was soluble in DMSO at a concentration of 100.0 mg/mL and was used to prepare primary 100X stock concentrations of CTFE trimer acid for use in the assay procedures. Treatment media were prepared by making 1:100 dilutions of the primary stocks into F12 tissue culture medium. Preliminary cytotoxicity testing showed the test material to be toxic to CHO cells both with and without S9 metabolic activation but only at the highest concentration tested (1.0 mg/mL).

Mutation assays were performed both with and without S9 metabolic activation. The test material produced toxicity in both mutation assays. The mutant frequency of treated cultures varied randomly with the dose within the range considered acceptable for background mutant frequencies (0 to 16.8×10^{-6}). No culture exposed to the test material had a mutant frequency that was statistically elevated over the mutant frequencies of the concurrent vehicle control cultures, with the exception of one culture from the assays conducted without S9 metabolic activation. The mutant frequency of this culture was elevated but was within the range considered acceptable for background mutant frequencies and was consistent with normal assay variation. Therefore, CTFE trimer acid was considered negative for inducing forward mutations at the HGPRT locus in CHO cells both with and without S9 metabolic activation.

INTRODUCTION

HGPRT is a cellular enzyme that permits cells to salvage hypoxanthine and guanine for use in DNA synthesis. The HGPRT enzyme utilizes the substrates 5-phosphoribosyl-1-pyrophosphate, hypoxanthine, or guanine to catalyze the formation of inosine or guanosine monophosphate. If a purine analog such as 6-thioguanine (TG) is included in the growth medium, the analog will be phosphorylated via the HGPRT pathway and incorporated into nucleic acids, eventually resulting in cellular death. The HGPRT locus is located on the X chromosome and because only one of the two X chromosomes is functional in the CHO cells, a single-step forward mutation from HGPRT+ to HGPRT- in the functional X chromosome renders the cell unable to utilize hypoxanthine, guanine, or TG supplied in the culture medium. These mutants are as viable as wild-type cells in normal medium because DNA synthesis may still proceed by *de novo* synthetic pathways that do not involve hypoxanthine or guanine as intermediates. The basis for the selection of HGPRT- mutants is the loss of their ability to utilize toxic purine analogs (e.g., TG), that enables only the HGPRT- mutants to grow in the presence of TG. Cells that grow to form colonies in the presence of TG are assumed to have mutated, either spontaneously or by the action of the test article, to the HGPRT- genotype.

MATERIALS AND METHODS

Test Material

CTFE trimer acid identified as ID# 10-86-63 and IR# 14240 was obtained from the U.S. Air Force. The clear, colorless, and slightly viscous liquid was stored at room temperature in the dark.

Media

The cells used during experimental studies were maintained in Ham's Nutrient Mixture F12 supplemented with L-glutamine, antibiotics, and fetal bovine serum (FBS, 8% v/v), hereafter referred to as culture medium. Cleansing medium used for reducing the spontaneous frequency of HGPRT- mutants prior to experimental studies consisted of culture medium supplemented with 5.0×10^{-6} M thymidine, 1.0×10^{-5} M hypoxanthine, 1.0×10^{-4} M glycine, 3.2×10^{-6} M of either aminopterin or methotrexate, and the concentration of FBS was reduced to 5% by volume. Recovery medium was similar to cleansing medium except that the aminopterin or methotrexate component was removed and the FBS was increased to 8% by volume. Selection medium for mutants was hypoxanthine-free F12 medium containing 4 μg/mL (24 mM) of TG and the FBS component reduced to 5% by volume.

Indicator Cells

The indicator cells used for this study were CHO cells originally derived from the ovary of a female Chinese hamster (Kao and Puck, 1968). Characteristics of the cell line are high clonability (approximately 85%) and rapid doubling time (11-14 h). The particular subclone used in this assay was CHO-K1-BH_4 that has been demonstrated to be sensitive to many chemical mutagens. Master stocks of the cells were maintained frozen in liquid nitrogen.

Laboratory cultures were maintained as monolayers at 37 ± 1.5°C in a humidified atmosphere containing 5 ± 1.5% CO_2 and were periodically checked for karyotype stability and for the absence of *Mycoplasma* contamination. To reduce the negative control frequency (spontaneous frequency) of HGPRT- mutants to the minimum possible, the cell cultures were exposed to conditions that selected against the HGPRT- phenotype. Cells were maintained in cleansing medium for two to three days, placed in recovery medium for one day, and then returned to culture medium. Cleansed cultures were used to initiate mutation assays from three to seven days after having been removed from cleansing medium.

Control Articles

A single negative (media) control culture was prepared for each portion of the rangefinding cytotoxicity assay by carrying cells unexposed to the test article through all of the assay operations. Negative controls were not prepared for the mutagenicity assay.

Vehicle controls were prepared for each portion of the assay by exposing cells to 1% DMSO in culture medium for 4 h. Single vehicle control cultures were used in the rangefinding cytotoxicity assay and duplicate vehicle controls were used in the mutagenicity assay.

5-Bromo-2'-deoxyuridine (BrdU) is highly and reproducibly mutagenic to CHO-K1-BH_4 cells without S9 metabolic activation. BrdU (Sigma Chemical Co.) was used at a concentration of 50 μg/mL of culture medium as a positive control article for mutagenicity assays conducted without S9 metabolic activation. 3-Methylcholanthrene (MCA, Sigma Chemical Co.) requires metabolic

activation by microsomal enzymes to become mutagenic to CHO-K1-BH_4 cells and was used at a concentration 5 μg/mL of culture medium as a positive control article for mutagenicity assays performed with S9 activation.

S9 Metabolic Activation System

The metabolic activation system was composed of rat liver enzymes and an energy producing reaction mixture prepared in a phosphate buffer (pH 7.8). The enzymes were contained in a 9000 x g supernatant (S9 fraction) from liver homogenate prepared from Sprague-Dawley rats treated with 500 mg/kg of Aroclor 1254 five days prior to sacrifice and purchased from Molecular Toxicology, Inc. The S9 fraction and reaction mixture were retained frozen at about -80°C until used. These components were thawed immediately before use and combined to form the metabolic activation system described below.

Activation System Component	Final Concentration in Cultures
NADP (sodium salt)	1.0 mM
Glucose-6-phosphate	5.0 mM
Calcium chloride	2.0 mM
Potassium chloride	6.6 mM
Magnesium chloride	2.0 mM
Phosphate	2.0 mM
S9 homogenate	15.0 - 20.0 μL/mL

The amount of S9 homogenate per culture was dependent upon the lot of S9 in use at any time. Before use each lot of S9 homogenate was tested in culture at various concentrations using chemicals such as benzo(a)pyrene or MCA. The optimum S9 concentration was selected based on induction of HGPRT-mutants in CHO cells exposed to these chemicals in culture, and this amount of S9 was used in all subsequent assays with that particular lot of S9.

Dosing Procedure

CTFE trimer acid was dissolved in DMSO at a concentration of 100 mg/mL which was 100 times the highest desired treatment concentration. Primary 100X test material stock solutions at lower concentrations were prepared by serial dilution with DMSO. Final 1X dosing solutions were prepared by making 1:100 dilutions of the primary stocks into culture medium containing 8% FBS for nonactivation studies and 5% FBS for activation studies. The volume of culture medium diluent in the activation studies was reduced to compensate for the volume of S9 reaction mixture used.

Preparations of test material in the vehicle were made fresh each day. Treatments were initiated by replacing the culture medium on the cell cultures with the treatment medium containing the test material at the desired concentrations.

Rangefinding Cytotoxicity Testing

A wide range of test article concentrations was tested for cytotoxicity both with and without S9 metabolic activation. Ten concentrations that ranged from 0.00195 to 1.0 mg/mL were used. In addition, one negative (media) control and one vehicle control containing 1% DMSO were used in each cytotoxicity assay.

The cells were quantitatively seeded at 200 cells per glass flask, allowed to attach overnight (16 to 18 h), and exposed to the test or control article for 4 h at 37 ± 1.5°C in a humidified atmosphere containing 5% CO_2. The cells were then washed twice with Dulbecco's phosphate buffered saline (PBS) and incubated in F12 culture medium for six additional days to allow for

colony development. Colonies were then fixed in alcohol, stained with Giemsa, and counted by eye, excluding those colonies with approximately 50 cells or less. Cytotoxicity was expressed as a percentage of colony counts in treated cultures versus control cultures. The preliminary cytotoxicity information was used to select doses for the mutation assay.

Nonactivation Mutagenicity Assay

This procedure was based on that reported by Hsie et al. (1975), reviewed by Hsie et al. (1981), with modifications suggested by Myhr and DiPaolo (1978). The cleansed cells were plated at approximately 2.4×10^6 cells per 75 cm^2 glass flask on the day before dosing. The time between plating and treatment was about 18 h. At that time cell cultures were treated with test or control material for 4 h at 37 ± 1.5°C in a humidified atmosphere with 5% CO_2. After treatment, the cell monolayers were washed twice with PBS, trypsinized, and suspended in culture medium. The cell suspension from cultures exposed to each dose was counted using a Coulter Counter and replated at 1.5×10^6 cells into each of two 150-mm dishes and at 200 cells into each of three 60-mm dishes. The small dishes were incubated for seven days to permit colony development and the determination of the cytotoxicity associated with each treatment. The larger dishes were incubated for seven days (the expression period) to permit growth and expression of induced mutations. The mass cultures were subcultured every two or three days during the expression period to maintain logarithmic cell growth. At each subculture the cells from the two 150-mm dishes from each dose were combined and reseeded at about 1.5×10^6 cells into each of two 150-mm dishes.

At the end of the expression period, each culture was reseeded at 2×10^5 cells per 100-mm dish (12 dishes total) in mutant selection medium. Three 60-mm dishes were seeded at 200 cells each in culture medium to determine the cloning efficiency (CE) of each culture. After incubation for seven to ten days, at 37 ± 1.5°C in a humidified atmosphere with 5% CO_2, the colonies were fixed with alcohol, stained with Giemsa, and counted to determine the number of TG-resistant colonies in mutant selection dishes and the number of colonies in the CE dishes. The colonies were counted by eye, excluding those with approximately 50 cells or less.

Activation Mutagenicity Assay

The activation assay was performed independently with its own set of vehicle and positive controls. The procedure was identical to the nonactivation assay except for the addition of the S9 fraction of rat liver homogenate and necessary cofactors during the 4-h treatment period. The FBS content of the medium used for dosing was reduced to 5% by volume.

Data Presentation

The raw data were used to calculate several assay parameters. The chosen combination of raw data and calculated data affords a complete description of events for each treatment condition. The significance of each calculated parameter and its method of calculation are listed below.

Relative Survival to Treatment: This parameter gives the clonal cytotoxicity of each treatment by showing what percentage of the cells were able to form colonies after the treatment period in both the rangefinding cytotoxicity assay and the mutagenicity assay relative to the concurrent vehicle controls. The average number of colonies in three dishes (seeded at 200 cells each) was determined for each treatment condition.

$$\text{Relative Survival (\%)} = \frac{\text{Average no. of colonies per treated culture}}{\text{Average no. of colonies per vehicle control dish}} \times 100\%$$

Relative Population Growth: This parameter shows the cumulative growth of the treated cell population, relative to the vehicle control growth, over the entire expression period and prior to mutant selection. In general, highly toxic treatments will reduce the growth rate as well as the survival.

Values less than 100% indicate growth inhibition. For example, 50% and 25% relative growth values would indicate treated cell populations that were one and two population doublings behind the vehicle control cultures. Treated populations that are more than 2 or 3 doublings behind the control might not achieve maximum expression of the TG-resistant phenotype. The relative population growth is calculated from cell count data not presented in this report and is intended to provide only an approximate indication of growth during the expression period, since cells are easily lost or not completely released by trypsin during the subculture procedures.

$$\text{Relative Population Growth (\%)} = \frac{\text{Treated culture population increase over the expression period}}{\text{Vehicle control population increase over the expression period}} \times 100\%$$

Absolute Cloning Efficiency: The ability of the cells to form colonies at the time of mutant selection is measured by the absolute CE. This parameter is used as the best estimate of the CE of the mutant cells in the selection dishes. Thus, the observed number of mutant colonies can be converted to the frequency of mutant cells in the treated population.

$$\text{Absolute CE (\%)} = \frac{\text{Average no. of viable colonies per dish}}{200} \times 100\%$$

Mutant Frequency: The mutant frequency is the endpoint of the assay. It is calculated as the ratio of colonies found in thioguanine selection medium to the total number of cells seeded, adjusted by the absolute CE. The frequency is expressed in units of 10^{-6}, e.g., the number of mutants per one million cells.

$$\text{Mutant Frequency (\%)} = \frac{\text{Total mutant clones}}{\text{no. of dishes} \times 2 \times 10^5 \times \text{absolute CE}} \times 100\%$$

RESULTS AND DISCUSSION

Test Article Handling

Solubility testing of the test material with DMSO revealed that good solubility was maintained at a concentration of 100.0 mg/mL. Dilutions were made with DMSO to prepare a series of 100X primary stocks at lower concentrations. Glass pipets and tubes were used to prepare primary test article stock solutions in DMSO.

Treatment media were prepared by making 1:100 dilutions of the test material stocks into F12 culture medium that contained 8% FBS for the nonactivation studies and 5% FBS for the S9 metabolic activation studies. Fresh primary test material stocks were prepared for each experiment. The cells were treated by replacing the media on the cultures with treatment media containing the different concentrations of test or control media.

The test material remained in solution in culture medium at concentrations ranging from 0.00195 mg/mL to the maximum applied concentration of 1.0 mg/mL. The test material did not alter the pH of the treatment medium outside the range of pH 7.0 to pH 7.8 at any applied concentration.

Rangefinding Cytotoxicity Assay

CTFE trimer acid was tested in the preliminary rangefinding cytotoxicity assay with and without S9 metabolic activation. Ten test article concentrations were used in each case ranging from 0.00195 to 1.0 mg/mL.

The rangefinding cytotoxicity assay showed that the test material was nontoxic to CHO cells in cultures at all but the highest dose both with and without S9 metabolic activation (Tables 4-1 and 4-2). While the absolute plating efficiencies (as measured by the number of colonies per culture dish) in the activation cytotoxicity assay appeared to be lower and more variable than normal, the data were sufficient to be used in selecting doses for the activation mutagenicity assay.

Mutagenicity Assay Without Metabolic Activation

Six test article concentrations were used ranging from 0.05 to 1.0 mg/mL. The test article was toxic to CHO cells only at a concentration of 1.0 mg/mL as measured by both relative clonal survival and relative population growth (Table 4-3). The mutant frequency of cultures treated with the test material varied within the acceptable range of vehicle control mutant frequency variation (0 to 16.8 x 10^{-6}). One of the six cultures had a mutant frequency that was significantly elevated over the mutant frequency of the vehicle control cultures, but this mutant frequency was within the range of acceptable background mutant frequencies and was consistent with normal assay variation (See Appendix 4-A). Therefore, CTFE trimer acid was evaluated as negative for inducing forward mutations at the HGPRT locus in CHO cells in the absence of S9 metabolic activation under the conditions of testing.

The positive control treatments with 50 μg/mL BrdU induced a large, significant ($p \leq 0.01$) increase in the mutant frequency. The mutant frequencies of the two vehicle controls were acceptable. Historical control mutant frequency data is presented in Appendix 4-A. The assay results achieved all assay acceptance criteria (Appendix 4-B), which provided confidence in the assumption that the recorded data represented a typical response of the test material in the nonactivation assay system.

Mutagenicity Assay With Metabolic Activation

CTFE trimer acid was tested in the presence of S9 metabolic activation using eight test article concentrations ranging from 0.05 to 1.0 mg/mL. The test article showed dose-related toxicity to CHO cells in culture as measured by both relative clonal survival and relative population growth (Table 4-4). The culture treated with 1.0 mg/mL CTFE trimer acid was excessively toxic and was not available for analysis. The seven remaining treated cultures were plated for mutant selection and were available for analysis. The mutant frequency of cultures treated with the test material varied randomly with dose within the acceptable range of vehicle control mutant frequency variation (0 to 10 x 10^{-6}) (See Appendix 4-A). There was no positive correlation of mutant frequency with dose and no treated culture had a mutant frequency that was significantly elevated over the average background mutant frequency of the concurrent vehicle controls. Therefore, CTFE trimer acid was evaluated as negative for inducing forward mutations at the HGPRT locus in CHO cells in the presence of S9 metabolic activation under the conditions of testing.

TABLE 4-1. CLONAL CYTOTOXICITY ASSAY OF CTFE TRIMER ACID WITHOUT METABOLIC ACTIVATION

Sample	Applied Concentration mg/mL	Number Colonies/Dish	Relative Survival[a] (%)	Cloning Efficiency (%)
NC[b]	------	149	97.4	
VC, 1%[c]	------	153	100.0	76.5
Trimer acid	0.00195	174	113.7	
Trimer acid	0.00391	168	109.8	
Trimer acid	0.00781	170	111.1	
Trimer acid	0.0156	159	103.9	
Trimer acid	0.0313	172	112.4	
Trimer acid	0.0625	168	109.8	
Trimer acid	0.125	174	113.7	
Trimer acid	0.25	159	103.9	
Trimer acid	0.5	150	98.0	
Trimer acid	1.0	60	39.2	

[a] Relative to 1% VC for all treatments.
[b] NC = Negative Control, F12 Medium.
[c] VC = Vehicle control, 1% DMSO.

TABLE 4-2. CLONAL CYTOTOXICITY ASSAY OF CTFE TRIMER ACID WITH METABOLIC ACTIVATION

Sample	Applied Concentration mg/mL	Number Colonies/Dish	Relative Survival[a] (%)	Cloning Efficiency (%)
NC[b]	-----	87	147.5	
VC, 1%[c]	-----	59	100.0	29.5
Trimer acid	0.00195	106	179.7	
Trimer acid	0.00391	118	200.0	
Trimer acid	0.00781	169	286.4	
Trimer acid	0.0156	67	113.6	
Trimer acid	0.0313	85	144.1	
Trimer acid	0.0625	70	118.6	
Trimer acid	0.125	50	84.7	
Trimer acid	0.25	96	162.7	
Trimer acid	0.5	135	228.8	
Trimer acid	1.0	0	0.0	

[a] Relative to 1% VC for all treatments.
[b] NC = Negative control, F12 Medium.
[c] VC = Vehicle control, 1% DMSO.

TABLE 4-3. MUTATION ASSAY OF CTFE TRIMER ACID WITHOUT METABOLIC ACTIVATION

Nonactivation Test Condition	Survival To Treatment (% Vehicle Control)	Relative Population Growth (% of Control)	Total Mutant Colonies	Absolute CE ± SD (%)	Mutant Freq in 10^{-6} Units[a]
Vehicle Control[b]	98.0	108.8	9	90.9 ± 7.3	4.1
Vehicle Control	102.0	91.2	9	91.5 ± 1.3	4.1
Positive Control[c]	69.7	41.1	237	97.7 ± 5.4	101.1[d]
Positive Control	61.6	43.7	299	91.7 ± 7.7	135.9[d]
CTFE (mg/mL)					
0.05	122.9	62.5	14	102.9 ± 8.4	5.7
0.1	100.1	63.3	13	113.4 ± 8.3	4.8
0.5	100.4	75.6	29	103.9 ± 6.4	11.6[d]
0.6	86.7	77.4	15	107.2 ± 3.8	5.8
0.8	96.0	73.0	7	94.4 ± 3.8	3.1
1.0	55.2	39.2	4	91.7 ± 5.0	1.8

[a] Mutant Frequency = Total mutant colonies/No. of dishes x 2 x 10^5 x absolute CE.
[b] Vehicle Control = 1% DMSO.
[c] Positive control = 50 µg/mL BrdU.
[d] Significant increase: Kastenbaum-Bowman test $p \leq 0.01$.

TABLE 4-4. MUTATION ASSAY OF CTFE TRIMER ACID WITH METABOLIC ACTIVATION

Aactivation Test Condition	Survival To Treatment (% Vehicle Control)	Relative Population Growth (% of Control)	Total Mutant Colonies	Absolute CE ± SD (%)	Mutant Freq in 10^{-6} Units[a]
Vehicle Control[b]	106.8	103.0	15	95.4 ± 5.6	6.6
Vehicle Control	93.2	97.0	14	103.2 ± 1.9	5.7
Positive Control[c]	136.5	144.8	432	100.5 ± 1.8	179.1[d]
Positive Control	152.1	128.7	428	104.0 ± 4.8	171.5[d]
CTFE (mg/ml)					
0.05	90.3	61.3	10	85.7 ± 4.8	4.9
0.1	70.5	54.5	5	97.0 ± 4.8	2.1
0.2	81.2	55.9	18	84.0 ± 1.3	8.9
0.3	52.6	41.2	14	87.5 ± 5.9	6.7
0.5	43.9	24.3	10	83.9 ± 7.0	5.0
0.6	42.6	29.6	22	92.0 ± 9.4	10.0
0.8	25.8	12.9	17	88.7 ± 1.2	8.0
1.0	3.5	NS[e]	-	-	-

[a] Mutant Frequency = Total mutant colonies/No. of dishes x 2 x 10^5 x absolute CE.
[b] Vehicle control = 1% DMSO.
[c] Positive control = 5.0 µg/mL MCA.
[d] Significant increase: Kastenbaum-Bowman test $p \leq 0.01$.
[e] NS = Not selected due to toxicity.

The positive control treatments with 5 μg/mL MCA induced large, significant ($p \leq 0.01$) increases in mutant frequency which demonstrated the effectiveness of the S9 metabolic activation system and the ability of the test system to detect known mutagens. The mutant frequencies of the vehicle controls were within the acceptable range (Appendix 4-A). The assay results achieved all assay acceptance criteria (Appendix 4-B) and provided confidence in the assumption that the recorded data represented typical responses of the test material in the assay system.

CONCLUSION

The test material, CTFE trimer acid, was considered negative for inducing forward mutations at the HGPRT locus in CHO cells under both the S9 metabolic activation and nonactivation conditions of the assay.

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APPENDIX 4-A

HISTORICAL CHO HGPRT ASSAY CONTROL MUTANT FREQUENCY DATA

A. Nonactivation Studies

1. Pooled negative and solvent controls

Mean (± SD)	$3.9 \pm 2.9 \times 10^{-6}$
Range	0 to 16.8×10^{-6}
Number of experiments	50
Number of controls	88

2. Positive controls (50 μg/mL 5-bromo-2'-deoxyuridine)

Mean (± SD)	$121.6 \pm 27.9 \times 10^{-6}$
Range	38.7 to 165.6×10^{-6}
Number of experiments	50
Number of controls	59

B. Activation Studies

1. Pooled negative and solvent controls

Mean (± SD)	$2.9 \pm 2.1 \times 10^{-6}$
Range	0 to 10.0×10^{-6}
Number of experiments	50
Number of controls	86

2. Positive controls (5 μg/mL 3-methylcholanthrene)

Mean (± SD)	$370.0 \pm 173.3 \times 10^{-6}$
Range	152.3 to 941.6×10^{-6}
Number of experiments	50
Number of controls	61

The historical control data was compiled from the most recent fifty experiments. Because some experiments contained duplicate controls, the number of independent control cultures exceeded the number of experiments.

APPENDIX 4-B

ASSAY ACCEPTANCE AND EVALUATION CRITERIA

Assay acceptance criteria

An assay normally is considered acceptable for evaluation of the results only if all of the following criteria are satisfied. The activation and nonactivation portions of the mutation assay may be performed concurrently, but each portion is, in fact, an independent assay with its own positive and vehicle controls. The activation or nonactivation assays will be repeated independently, as needed, to satisfy the acceptance and evaluation criteria.

The average absolute cloning efficiency of the vehicle controls should be between 70% and 115%. A value greater than 100% is possible because of errors in cell counts (usually ± 10%) and dilutions during cloning. Cloning efficiencies below 70% do not necessarily indicate substandard culture conditions or unhealthy cells. Assay variables can lead to artificially low cloning efficiencies in the range of 50 to 70% and still yield internally consistent and valid results. Assays with cloning efficiencies in this range will be conditionally acceptable and dependent on scientific judgment. All assays below 50% cloning efficiency will be unacceptable.

The background mutant frequency (average of the vehicle controls) is calculated separately for the activation and nonactivation assays, even though the same population of cells may be used for concurrent assays. The activation vehicle controls contain the S9 activation mix and may have a slightly different mutant frequency than the nonactivation vehicle controls. For both conditions, background frequencies for assays performed with different cell stocks are generally 0 to 10 x 10^{-6}. Assays with backgrounds greater than 15 x 10^{-6} will not be used for evaluation of a test article.

A positive control is included with each assay to provide confidence in the procedures used to detect mutagenic activity. An assay will be acceptable in the absence of a positive control (loss due to contamination or technical error) only if the test article clearly shows mutagenic activity as described in the evaluation criteria. If the test article appears to have no or only weak mutagenic activity, an acceptable assay must have a positive control mutant frequency that is significantly elevated over the concurrent vehicle controls ($p \leq 0.01$).

For test articles with little or no mutagenic activity, an acceptable assay should include applied concentrations that reduce the clonal survival to approximately 10% to 15% of the average of the vehicle controls, reach the maximum applied concentrations given in the evaluation criteria, reach a concentration that is approximately twice the solubility limit of the test article in culture medium, or include a high concentration that is at least 75% of an excessively toxic concentration. There is no maximum toxicity requirement for test articles which clearly show mutagenic activity.

Mutant frequencies are normally derived from sets of twelve dishes for the mutant colony count and three dishes for the viable colony count. To allow for contamination losses, an acceptable mutant frequency for treated cultures can be calculated from a minimum of eight mutant selection dishes and two cloning efficiency dishes.

The mutant frequencies for five treated cultures are normally determined in each assay. A required number of different concentrations cannot be explicitly stated, although a minimum of three analyzed cultures is considered necessary under the most favorable test conditions in order to accept a single assay for evaluation of the test article.

Assay evaluation criteria

Mutation assays are initiated by exposing cell cultures to about six to eight concentrations of test article that are expected, on the basis of preliminary toxicity studies, to span a range of cellular responses from no observed toxicity to about 10% survival. Five doses are usually then selected for completion of the mutation assay. These doses should cover a range of toxicities with emphasis placed on the most toxic doses. An assay may need to be repeated with different concentrations to properly evaluate a test article.

The statistical tables provided by Kastenbaum and Bowman (1970) are used to determine whether the results at each dose are significantly different from the negative controls at 95% or 99% confidence levels. This test compares variables distributed according to Poissonian expectations by summing up the probabilities in the tails of two binomial distributions. The 95% confidence level must be met as one criterion for considering the test article to be active at a particular dose. In addition, the mutant frequency must meet or exceed 15×10^{-6} in order to compensate for random fluctuations in the 0 to 10×10^{-6} background mutant frequencies that are typical for this assay.

Observation of a mutant frequency that meets the minimum criteria for a positive response in a single treated culture within a range of assayed concentrations is not sufficient evidence to evaluate a test article as a mutagen. The following test results must be obtained to reach this conclusion for either activation or nonactivation conditions:

A dose-related or toxicity-related increase in mutant frequency should be observed. It is desirable to obtain this relation for at least three doses. However, this depends on the concentration steps chosen for the assay and the toxicity at which mutagenic activity appears. If an increase in mutant frequency is observed for a single dose near the highest testable toxicity, as defined previously, and the number of mutant colonies is more than twice the value needed to indicate a significant response, the test article generally will be considered mutagenic. Smaller increases at a single dose near the highest testable toxicity will require confirmation by a repeat assay.

For some test articles, the correlation between toxicity and applied concentration is poor. The proportion of the applied article that effectively interacts with the cells to cause genetic alterations is not always repeatable or readily controlled. Conversely, measurable changes in the frequency of induced mutants may occur with concentration changes that cause only small changes in observable toxicity. Therefore, either parameter, applied concentration, or toxicity (percent survival), can be used to establish whether the mutagenic activity is related to an increase in effective treatment.

Treatments that reduce relative clonal survival to less than five percent may be included in the assay but will not be used as sufficient evidence for mutagenicity as it relates to risk assessment.

A test article is evaluated as nonmutagenic in a single assay only if the minimum increase in mutant frequency is not observed for a range of applied concentrations that extends to concentrations causing about 10% to 15% survival or extends to a concentration at least 75% of that causing excessive toxicity. If the test article is relatively nontoxic, the maximum applied concentration will normally be 5 mg/mL (or 5 μL/mL) for water-soluble

materials or 1 mg/mL (or 1 μL/mL) for materials in organic solvents. If a repeat assay does not confirm an earlier, minimal response as discussed above, the test article is evaluated as nonmutagenic in this assay system.

This presentation may not encompass all test situations, and criteria may be used to arrive at a conclusion, especially when data from several repeat assays are available. The interpretation of the results in the Results and Discussion section provides the reasoning involved when departures from the above descriptions occur.

SECTION 5

MUTAGENICITY TEST ON CTFE TRIMER ACID IN AN *IN VITRO* CYTOGENETIC ASSAY MEASURING SISTER CHROMATID EXCHANGE AND CHROMOSOMAL ABERRATION FREQUENCIES IN CHINESE HAMSTER OVARY CELLS

H. Murli

ABSTRACT

The objective of this assay was to evaluate the ability of CTFE trimer acid to induce SCE and chromosomal aberrations in CHO cells both with and without S9 metabolic activation. In the SCE assay, duplicate cultures of CHO cells were incubated with concentrations of CTFE trimer acid ranging from 0.167 to 5020 μg/mL in a half-log series. There was no significant increase in SCE at the concentrations tested.

Cell cycle kinetics evaluated from the SCE assay indicated cell cycle delay at concentrations of 167 and 502 μg/mL under nonactivation conditions. The addition of S9 microsomal enzymes caused a slight cell cycle delay at concentrations greater than 1670 μg/mL. Based on these evaluations of cell cycle kinetics, duplicate cultures of CHO cells were tested with concentrations ranging from 49.8 to 99.6 μg/mL of CTFE trimer acid in a 10-h aberrations assay and from 101 to 1010 μg/mL in a 20-h aberrations assay under nonactivation conditions, and with 249 to 4980 μg/mL in a 10-h aberrations assay with S9 metabolic activation. No increase in cells with chromosomal aberrations was observed at the concentrations analyzed.

The test article, CTFE trimer acid, was considered negative for inducing SCE and negative for inducing chromosomal aberrations in CHO cells both with and without metabolic activation.

INTRODUCTION

The chromosomes of dividing cells consist of two identical halves, or sister chromatids. SCEs are seen at metaphase of cell division as reciprocal interchanges of the two chromatid arms within a single chromosome. These exchanges presumably require enzymatic incision, translocation, and ligation of the two DNA strands. The frequency of SCEs is thought to be a very sensitive indicator of damage to DNA and are caused by many chemical agents known to be mutagens or carcinogens.

The SCE assay involves treating cultured cells with a test compound, exposing the cells in culture to the thymidine analog BrdU for two cell cycles, and making chromosome preparations that are stained for SCE. By growing cells with BrdU for two cell cycles one chromatid contains half as much BrdU as the other and is stained more intensely by Giemsa, while its pair (the sister chromatid), is pale.

The chromosomal aberrations assay was designed to examine cells in the first mitosis after chemical exposure to establish whether the test article and/or its metabolites can interact with cells to induce chromosomal breaks. Aberrations are a consequence of failure or mistakes in repair processes resulting in breaks that do not rejoin or rejoin in abnormal configurations (Evans, 1962). Examining cells in the first mitosis after chemical exposure limits loss of aberrant cells during the division process or conversion into complex derivatives during subsequent cell cycles. In the case of CHO cells most dividing cells examined 8-12 h after treatment are in the first mitosis (M_1 cells). However, many test articles cause severe delay of progression

through the cell cycle, and the assay has been designed to detect this delay and allow for slower growth of damaged cells by adjustments in the time between treatment and cell fixation.

EXPERIMENTAL DESIGN

In the SCE assay, CHO cell cultures that were exposed to the test article for approximately two cell cycles were analyzed to determine cellular toxicity and effects of the test article on cell generation time. If necessary and possible, the assay was extended in cultures at affected doses to permit the progression to second generation cells. The doses used in the assay ranged from 0.167 to 5020 μg/mL of the test material in a half-log series. Single cultures were used for the negative control, solvent control, and each of two doses for the positive control; duplicate cultures were used for the ten doses of the test material. SCE frequencies were analyzed from cultures treated with the four highest doses having second generation cells and from a negative, solvent, and positive control culture. Cell cycle kinetics of the treated cultures were also evaluated. A summary of the treatment schedule for the SCE assay is given below.

SUMMARY OF SCE ASSAY TREATMENT SCHEDULE IN HOURS (APPROXIMATE)

Test	Test Article	Wash	BrdU	Wash	Colcemid	Fixation
-S9	-2.25		0	22.75	23	25.5
+S9	-2.25	-0.25	0		23	25.5

Cell cycle kinetics from the SCE assay were used to determine the dose range to be used in the chromosomal aberrations assay and to determine the optimal time of harvest of the treated cells so that primarily metaphase cells (in the first metaphase since exposure to the test article) would be analyzed for chromosomal aberrations. The aberrations assay was conducted at the 10-h harvest time for those chemicals that did not induce any cell cycle delay and at the 20-h harvest time for those chemicals that induced cell cycle delay.

In the chromosomal aberrations assay duplicate cultures were used for each dose. Single cultures were used for the negative control, solvent control, and at each of two doses for the positive control. In the nonactivation assay, 10- and 20-h harvests were conducted while a 10-h harvest was conducted in the activation assay. Chromosomal aberrations were analyzed for the cultures treated at the four highest doses from which results could be obtained and from only one of the positive control doses. A summary of the treatment schedule for the chromosomal aberrations assay is given below.

SUMMARY OF CHROMOSOMAL ABERRATIONS ASSAY TREATMENT SCHEDULE IN HOURS (APPROXIMATE)

Test	Test Article	Wash	Colcemid	Fixation
-S9	0	7.25	7.5	10
-S9	0	17.2	17.5	20
+S9	0	2	7.5	10

MATERIALS AND METHODS

Test Material

The CTFE trimer acid used in these studies was provided by the U.S. Air Force. The clear, colorless, and slightly viscous liquid was further identified as ID# 10-86-63, IR# 14240 and stored in the dark.

Indicator Cells

The CHO cells (CHO-WBL) used in this assay were from a permanent cell line and have been recloned to maintain karyotypic stability. This cell line has an average cycle time of 12 to 14 h with a modal chromosome number of 21.

Cell Culture Medium

The CHO cells were grown in McCoy's 5a culture medium that was supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin and streptomycin at 37°C in an atmosphere of 5% CO_2 in air.

Negative and Solvent Controls

In the nonactivation assays, negative controls were cultures that contained only cells and culture medium. Solvent controls were cultures containing the solvent in place of the test article at the same concentration used in test cultures. In the activation assays, the negative and solvent controls were the same as in the nonactivation assays but the S9 activation mixture was included.

Positive Control Agents

The positive control agents that were used in the assays were mitomycin C (MMC) for the nonactivation assays and cyclophosphamide (CP) for the metabolic activation assays. MMC is a clastogen that does not require metabolic activation, while CP must first be converted to active metabolites by microsomal enzymes. In the SCE assay two doses of MMC (0.005 μg/mL and 0.010 μg/mL) and CP (1.50 μg/mL and 2.00 μg/mL) were used. In the chromosomal aberrations assays two concentrations of MMC (0.500 μg/mL and 1.00 μg/mL, 10-h harvest; 0.040 μg/mL and 0.080 μg/mL, 20-h harvest) and CP (25.0 μg/mL and 50.0 μg/mL) were used. Only cultures exposed to one dose of the positive control were actually analyzed in each of the SCE and aberration assays.

Sister Chromatid Exchange Assays

In these assays, the cells were cultured for approximately 24 h prior to treatment by seeding approximately 0.8 x 10^6 cells per 75 cm^2 flask into 10 mL of complete McCoy's 5a culture medium. BrdU was added at a final concentration of 10 μM approximately 2 h after the initial exposure of the cells to the test article.

Nonactivation Assay: The cultures were dosed with the test article for approximately 2.5 hours and then BrdU was added at a final concentration of 10 μM. The cultures were reincubated for approximately 23 h. Approximately 2.75 h prior to the harvest of the cells, the test article was washed from the cells with PBS and fresh culture medium containing BrdU (10 μM) and Colcemid (final concentration 0.1 μg/mL) was added.

Prior to the harvest of the cultures visual observations of toxicity were made. These observations included an assessment of the percent confluence of the cell monolayer within the culture flasks. The cultures were also evaluated for the presence of mitotic (large rounded cells) or dead cells

floating in the medium. Only flasks from the highest seven surviving doses from which metaphase cells for analysis were expected were harvested (See Section on Harvest).

The culture medium was collected and centrifuged. The mitotic cells were fixed and fresh culture medium was replaced on the remaining cell monolayer in the flasks. A test slide was made from fixed cells treated with the highest doses of test compound and stained with Hoechst 33258 stain (0.5 μg/mL in PBS, pH 6.8). The slides were examined under ultraviolet (UV) fluorescence microscopy. If there was a marked cell cycle delay, a second cell collection was made following an additional 6 h and mitotic cells were harvested. These harvested cells were differentially stained for the analysis of SCE using a modified fluorescence-plus-Giemsa (FPG) technique (See Sections on Harvest and Slide Preparation and Staining).

Assay with Metabolic Activation: In this assay, the CHO cells were exposed to the test article for 2 h in the presence of a rat liver S9 reaction mixture (S9 15 μL/mL, NADP 1.5 mg/mL, and isocitric acid 2.7 mg/mL). The S9 fraction was derived from the livers of male Sprague-Dawley rats treated with Aroclor 1254 to induce the mixed function oxidase enzymes and purchased from Molecular Toxicology Inc. The 2-h incubation time was chosen because prolonged exposure to the S9 mixture can be toxic to the cells and enzyme activity of S9 is lost rapidly at 37°C. The medium did not contain FBS during the exposure period because highly reactive or short-lived intermediates produced by the S9 enzymes can bind to serum proteins.

After the exposure period the cells were washed twice with PBS. Complete McCoy's 5a medium containing 10 μM BrdU was added to the cultures that were then incubated for approximately 23 h. Colcemid (final concentration 0.1 μg/mL) was then added, the cultures reincubated for 2.5 h, and the cells harvested and examined for any cell cycle delay. Slides were prepared and stained as described for the nonactivation assay. Delayed fixation was not required for any of the surviving cultures.

Chromosomal Aberrations Assay

Nonactivation Assay: Cultures were initiated by seeding approximately 1.0×10^6 cells (20-h assay) and 1.2×10^6 cells (10-h assay) per 75 cm^2 flask into 10 mL of complete McCoy's 5a medium. One day after culture initiation, the CHO cells to be used in the nonactivation assay were treated with the test article at predetermined doses for 7.25 and 17.25 h. The cultures were then washed with PBS and complete medium containing 0.1 μg/mL Colcemid was added back to the cultures. Two and one-half hours later the cells were harvested and air-dried slides were prepared and stained in 5% Giemsa solution for the analysis of chromosomal aberrations.

Assay with Metabolic Activation: Cultures were initiated by seeding approximately 1.2 x 10 cells per 75 cm flask into 10 mL of complete McCoy's 5a medium. One day after culture initiation, the cultures were incubated at 37°C for 2 h in the presence of the test article and the S9 reaction mixture in McCoy's 5a medium without FBS. After the 2-h exposure period the cells were washed twice with PBS and complete medium was added back to the cultures. The cells were incubated for an additional 7.75 h with 0.1 μg/mL Colcemid added during the last 2.5 h of incubation. The metaphase cells were then harvested and prepared for cytogenetic analysis.

Harvest Procedure

The metaphase cells were collected by mitotic shake-off (Terasima and Tolmach, 1961) and were treated with 0.075 M KCl solution to swell the cells and disperse the chromosomes. The cultures were then fixed with an absolute methanol/glacial acetic acid (3:1, v/v) fixative and were washed several times before air-dried slides were prepared.

Slide Preparation and Staining

Slides were prepared by dropping the harvested cultures on clean slides. The slides from the rangefinding assays were differentially stained using a modified FPG technique (after Perry and Wolff, 1974; Goto et al., 1978). The slides were stained in Hoechst 33258 stain, exposed to UV light, and stained with Giemsa Azure B stain. Slides for analysis of chromosomal aberrations were stained with 5% Giemsa solution. All slides were then air-dried and coverslipped using Depex mounting medium.

SCE Analysis and Assay Evaluation

Fifty cells per dose were analyzed from each of the top four doses from which sufficient M_2 metaphase cells were available. Fifty cells were analyzed from each of the negative and solvent controls, and at least twenty cells were analyzed from one of the positive control doses. For control of bias, all slides except for the positive controls were coded prior to analysis. Cells were selected for scoring on the basis of good morphology and clear sister chromatid differentiation along the entire length of all chromosomes; only cells with the number of centromeres equal to the modal number 21 ± 2 (range of 19-23) were analyzed.

The slides were also examined for the presence of delayed cells. One hundred metaphase cells were scanned and classified as M_1, M_{1+}, or M_2 from each dose and the positive, negative, and solvent controls to give an estimate of cell cycle inhibition. In those doses where more than one harvest was conducted, cells were analyzed for cell cycle kinetics and SCE from the earliest harvest time from which sufficient M_2 cells were available for analysis. Controls were analyzed only at the normal harvest time (25-26 h). The cell cycle kinetics were presented as replicative index (RI), calculated using the formula $(1 \times M_1 + 1.5 \times M_{1+} + 2 \times M_2 + 2.5 \times M_{2+})$/number of cells analyzed (Schneider et al., 1981).

If an increase in SCE was observed, one of the following criteria must have normally been met to assess the compound as positive.

Two-fold increase: Approximately a doubling in SCE frequency over the "background" (solvent and negative control) levels at one or more doses.

Dose response: A positive assessment may be made in the absence of a doubling of the SCE frequency if there was a statistically significant increase at a minimum of three doses and evidence for a positive dose response.

In some cases, a statistically significant increase was observed with neither a doubling nor a dose response. These results were assessed according to the magnitude of the response, and the proportion of the doses affected.

Statistical analysis employed a Dunnett's one-tailed t-test (Bancroft, 1957; Hollander and Wolfe, 1973) to compare SCE frequencies in the tested cultures with that of the negative and solvent controls. The final evaluation of the test article was based upon scientific judgment.

Aberrations Analysis and Assay Evaluation

Cells were selected for good morphology and only cells with the number of centromeres equal to the modal number 21 ± 2 (range 19-23) were analyzed. One hundred cells, if possible, from each duplicate cultures at four doses of the test article and from each of the negative and solvent control cultures were analyzed for the different types of chromosomal aberrations (Evans, 1962). At least 25 cells were analyzed for chromosomal aberrations from one of the positive control cultures. For control of bias, all slides except for the positive controls were coded prior to analysis.

The following factors were taken into account in the evaluation of the chromosomal aberrations data:

The overall chromosomal aberration frequencies.
The percentage of cells with any aberrations.
The percentage of cells with more than one aberration.
Any evidence for increasing amounts of damage with increasing dose, i.e., a positive dose response.
The estimated number of breaks involved in the production of the different types of aberrations which were observed, i.e., complex aberrations may have more significance than simple breaks.

Chromatid and isochromatid gaps, if observed, were noted in the raw data and were tabulated. They were not, however, considered in the evaluation of the ability of the test article to induce chromosomal aberrations since they may not represent true chromosomal breaks and may possibly be induced by toxicity.

A cell classified as "GT" was considered to contain 10 aberrations for statistical purposes but a ">" was also included in the tables for this classification to indicate that it was a minimum number.

Statistical analysis employed the Fisher's Exact Test with an adjustment for multiple comparisons (Sokal and Rohlf, 1981) to compare the percentage of cells with aberrations in each treatment group with the results from the pooled solvent and negative controls (the solvent and negative controls were statistically evaluated for similarity prior to the pooled evaluation). Test article significance was established where $p \leq 0.01$. All factors as stated previously were taken into account and the final evaluation of the test article was based upon scientific judgment.

RESULTS AND DISCUSSION

Solubility, Stability, and Dose Determination

CTFE trimer acid was soluble in DMSO and formed a clear colorless solution at a concentration of 502 mg/mL. A half-log series of concentrations from 0.167 to 5020 µg/mL was tested in the SCE assay.

SCE Assay

Nonactivation Assay: A precipitate was observed at a dose of 5020 µg/mL and complete cytotoxicity was observed at doses of 1670 and 5020 µg/mL. A slight reduction in the number of visible mitotic cells and an approximately 15% reduction in the cell monolayer confluence were observed at a dose of 502 µg/mL. Fluorescent examination of the cells exposed to doses of 167 and 502 µg/mL indicated the presence of some cell cycle delay. These cultures were incubated for six more hours and then harvested. Sufficient M_2 cells were not available from cultures exposed to 502 µg/mL, even after the delayed harvest, and only a sparse number of metaphase cells were available for analysis. Results were analyzed from cultures exposed to concentrations of

5.02, 16.7, 50.2, and 167 μg/mL and are presented in Table 5-1. No significant increase in SCE was observed at the concentrations analyzed. The sensitivity of the cell culture for induction of SCE was demonstrated by the increased frequency of SCE in the cells exposed to the positive control agent. CTFE trimer acid was considered negative for inducing SCE under conditions of nonactivation.

Assay With Metabolic Activation: A precipitate was observed after dosing and complete cytotoxicity was observed prior to harvest at a dose 5020 μg/mL. A slight reduction in the number of visible mitotic cells and an approximately 15% reduction in the cell monolayer confluence was observed at a dose of 1670 μg/mL. Fluorescent examination of the cells on the slides prepared at a dose of 1670 μg/mL indicated no cell cycle delay. Results were analyzed from cultures exposed to 50.2, 167, 502, and 1670 μg/mL and are presented in Table 5-2. A weakly significant increase in SCE was observed at a dose of 1670 μg/mL. There was no apparent dose response and the observed increase in SCE was very slight and was within the limits of historical control data for DMSO (6.46 to 10.90). Therefore, the increase was not considered significant. The successful activation of the metabolic system was demonstrated by the increased frequency of SCE in the cells induced with the positive control agent. The test article was therefore considered negative for inducing SCE under conditions of metabolic activation.

TABLE 5-1. SISTER CHROMATID EXCHANGE IN CHO CELLS WITHOUT METABOLIC ACTIVATION OF CTFE TRIMER ACID

	Dose μg/mL	Total Cells Scored	# of Chromo-somes	# of SCE	SCE/ Chromo-some	SCE/Cell Mean ± SE	RI	% SCE Increase Over Solvent	Confluence % Solvent Control
McCoys 5a	-----	50	1038	362	0.35	7.24 ± 0.32	1.91		
DMSO[a]	-----	50	1032	394	0.38	7.88 ± 0.34	1.75		100
MMC	0.005	20	412	553	1.34	27.65 ± 1.62*	1.84	252	100
TRIMER ACID	5.02	50	1041	413	0.40	8.26 ± 0.40	1.71	4	100
TRIMER ACID	16.7	50	1036	372	0.36	7.44 ± 0.33	1.76		100
TRIMER ACID	50.2	50	1040	395	0.38	7.90 ± 0.34	1.78		100
TRIMER ACID	167	50	1033	436	0.42	8.72 ± 0.36	1.46	11	100
TRIMER ACID	502**						1.13		86

[a] Concentration of DMSO = 1% v/v.

* Significantly greater than the solvent control, $p < 0.05$.

**Toxic dose.

TABLE 5-2. SISTER CHROMATID EXCHANGE IN CHO CELLS WITH METABOLIC ACTIVATION OF CTFE TRIMER ACID

	Dose µg/mL	Total Cells Scored	# of Chromo-somes	# of SCE	SCE/ Chromo-some	SCE/Cell Mean ± SE	RI	% SCE Increase Over Solvent	Confluence % Solvent Control
McCoys 5a	-----	50	1037	370	0.36	7.40 ± 0.32	1.86		
DMSO[a]	-----	50	1040	421	0.40	8.42 ± 0.45	1.87		100
CP	1.50	20	411	633	1.54	31.65 ± 2.13*	1.77	280	100
TRIMER ACID	50.2	50	1039	434	0.42	8.68 ± 0.35	1.88	3	100
TRIMER ACID	167	50	1042	384	0.37	7.68 ± 0.37	1.81		100
TRIMER ACID	502	50	1035	447	0.43	8.94 ± 0.40	1.81	7	100
TRIMER ACID	1670	50	1039	483	0.46	9.66 ± 0.43*	1.85	15	86
TRIMER ACID	5020**								<7

[a] Concentration of DMSO = 1% v/v.

* Significantly greater than the solvent control, $p < 0.05$.

**Toxic dose.

Chromosomal Aberrations Assay

Nonactivation assay: Because severe cell cycle delay was observed at doses of 167 and 502 µg/mL (Table 5-1) a 10-h harvest was conducted in the nonactivation aberration assay that tested doses of 49.8, 74.7, and 99.6 µg/mL, and a 20-h harvest was conducted that tested doses of 101, 254, 507, 761, and 1010 µg/mL.

In the 10-h assay, a slight reduction in the number of visible mitotic cells was observed at doses of 74.7 and 99.6 µg/mL. Since four doses were available for analysis from the 20-h assay, these cultures were not analyzed.

In the 20-h assay floating dead cells, a severe reduction in the number of visible mitotic cells, and an approximately 30% reduction in the cell monolayer confluence was observed at a dose of 1010 µg/mL. A reduction in the number of visible mitotic cells was observed at a dose of 761 µg/mL, and a slight reduction in the number of visible mitotic cells was observed at a dose of 507 µg/mL. Results were analyzed from cultures exposed to doses of 254, 507, 761, and 1010 µg/mL (results pooled from the replicate cultures are presented in Table 5-3, and results from individual cultures are presented in Table 5-4). No significant increase in cells with chromosomal aberrations was observed at the concentrations analyzed. The sensitivity of the cell culture for induction of chromosomal aberrations was demonstrated by the increased frequency of aberrations in the cells exposed to the positive control agent. The test article was therefore considered negative for inducing chromosomal aberrations under nonactivation conditions.

Assay With Metabolic Activation: Only insignificant cell cycle delay was observed at the doses analyzed (Table 5-2). Based on these cell cycle kinetics, a 10-h harvest was conducted that tested doses of 249, 374, 498, 1250, 2490, 3740, and 4980 µg/mL.

A slightly cloudy cell culture medium, an unhealthy cell monolayer, an approximately 75% reduction in the cell monolayer confluence, floating dead cells and debris, and a severe reduction in the number of visible mitotic

cells was observed at a dose of 4980 μg/mL. Only dead cells were available on the slides prepared from these cultures. An unhealthy cell monolayer, an approximately 75% reduction in the cell monolayer confluence, floating dead cells and debris, and a severe reduction in the number of visible mitotic cells was observed at a dose of 3740 μg/mL, and again only dead cells were available on the slides prepared from these cultures. An unhealthy cell monolayer, an approximately 50% reduction in the cell monolayer confluence, and a reduction in the number of visible mitotic cells were observed at a dose of 2490 μg/mL. Results were analyzed from cultures exposed to 374, 498, 1250, and 2490 μg/mL (results pooled from the replicate cultures are presented in Table 5-5, and results from individual cultures are presented in Table 5-6). No significant increase in cells with chromosomal aberrations was observed at the doses analyzed, except at a dose of 2490 μg/mL. This dose induced severe toxicity and only 56 and 75 cells, respectively, were available from the replicate cultures. The increase observed in one of the cultures was weak and was not observed in the other. Thus the increase was probably due to the severe cytotoxicity, rather than true clastogenicity. The successful activation of the metabolic system was illustrated by the increased incidence of cells with chromosomal aberrations in the cultures induced with CP, the positive control agent. The test article was considered negative for inducing chromosomal aberrations under conditions of metabolic activation.

TABLE 5-3. CHROMOSOME ABERRATIONS IN CHO CELLS WITHOUT METABOLIC ACTIVATION OF CTFE TRIMER ACID (RESULTS POOLED FROM DUPLICATE CULTURES)

		Number and Type of Aberration																
		Not Computed			Simple				Complex						Other			
	Cells Scored	TG	SG	UC	TB	SB	DM	ID	TR	QR	D	R	CI	GT	No. of Aberrations Per Cell	% Cells with Aberrations	% Cells with >1 Aberration
Media + DMSO	200	7	2			2					1				0.02	1.5	0.0
MMC (0.040 μg/mL)	25	3	2		4	4			3	4					0.60	52.0*	8.0*
Test Article																	
254 μg/mL	200	6	4			1									0.01	0.5	0.0
507 μg/mL	200	6	1			1					1				0.01	1.0	0.0
761 μg/mL	200	10	2			1									0.01	0.5	0.0
1010 μg/mL	200	9	3		1	5			1		1				0.04	2.5	0.5

* Significantly greater than the pooled negative and solvent controls, $p < 0.01$.

TABLE 5-4. CHROMOSOME ABERRATIONS IN CHO CELLS WITHOUT METABOLIC ACTIVATION OF CTFE TRIMER ACID (RESULTS FROM INDIVIDUAL CULTURES)

		Cells Scored	Number and Type of Aberration: Not Computed TG	SG	UC	Simple TB	SB	DM	ID	Complex TR	QR	D	R	CI	Other GT	No. of Aberrations Per Cell	% Cells with Aberrations	% Cells with >1 Aberration
Controls																		
McCoy's 5a		100	3				2									0.02	2.0	0.0
1% v/v DMSO		100	4	2								1				0.01	1.0	0.0
MMC (0.040 µg/mL)		25	3	2		4	4			3	4					0.60	52.0*	8.0*
Test Article																		
254 µg/mL	A	100	2	3												0.00	0.0	0.0
	B	100	4	1			1									0.01	1.0	0.0
507 µg/mL	A	100	2	1												0.00	0.0	0.0
	B	100	4				1					1				0.02	2.0	0.0
761 µg/mL	A	100	5				1									0.01	1.0	0.0
	B	100	5	2												0.00	0.0	0.0
1010 µg/mL	A	100	4	2												0.00	0.0	0.0
	B	100	5	1		1	5			1		1				0.08	5.0	1.0

* Significantly greater than the pooled negative and solvent controls, p <0.01.

TABLE 5-5. CHROMOSOME ABERRATIONS IN CHO CELLS WITH METABOLIC ACTIVATION OF CTFE TRIMER ACID (RESULTS POOLED FROM DUPLICATE CULTURES)

	Cells Scored	Number and Type of Aberration: Not Computed TG	SG	UC	Simple TB	SB	DM	ID	Complex TR	QR	D	R	CI	Other GT	No. of Aberrations Per Cell	% Cells with Aberrations	% Cells with >1 Aberration
Media and DMSO	200	2	2			1									0.01	0.5	0.0
CP (25µg/mL)	25	5			2	8			3						0.52	28.0*	16.0*
Test Article																	
374 µg/mL	200	9	2			1					1				0.01	1.0	0.0
498 µg/mL	200	5	3			1					1				0.01	1.0	0.0
1250 µg/mL	200	4	1												0.00	0.0	0.0
2490 µg/mL**	131	17	7	1	2	3			2	2	1				0.08	6.1*	1.5
3740 µg/mL***																	

* Significantly greater than the pooled negative and solvent controls, p < 0.01.

** Nearly toxic dose. Only 131 cells available for analysis from the replicate cultures.

***Toxic dose.

TABLE 5-6. CHROMOSOME ABERRATIONS IN CHO CELLS WITH METABOLIC ACTIVATION OF CTFE TRIMER ACID (RESULTS FROM INDIVIDUAL CULTURES)

			Number and Type of Aberration															
			Not Computed		Simple				Complex					Other				
		Cells Scored	TG	SG	UC	TB	SB	DM	ID	TR	QR	D	R	CI	GT	No. of Aberrations Per Cell	% Cells with Aberrations	% Cells with >1 Aberration
McCoy's 5a		100	2	2												0.00	0.0	0.0
DMSO 1% v/v		100					1									0.01	1.0	0.0
CP (25µg/mL)		25	5			2	8			3						0.52	28.0*	16.0*
Test Article																		
374 µg/mL	A	100	2	1												0.00	0.0	0.0
	B	100	7	1						1		1				0.02	2.0	0.0
498 µg/mL	A	100	3	1												0.00	0.0	0.0
	B	100	2	2			1					1				0.02	2.0	0.0
1250 µg/mL	A	100	4													0.00	0.0	0.0
	B	100		1												0.00	0.0	0.0
2490 µg/mL	A**	56	5	1	1		1			1						0.04	3.6	0.0
	B**	75	12	6		2	2			1	2	1				0.11	8.0	2.7
3740 µg/mL***																		

* Significantly greater than the pooled negative and solvent controls, $p < 0.01$.

** Nearly toxic dose. Only 56 and 75 cells available for analysis from the replicate cultures.

***Toxic dose.

CONCLUSION

CTFE trimer acid was considered negative for inducing SCE and chromosomal aberrations in CHO cells under both the metabolic activation and nonactivation conditions of this assay.

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APPENDIX 5-A

DEFINITIONS OF CHROMOSOME ABERRATIONS FOR GIEMSA STAINED CELLS

NOT COMPUTED

TG	Chromatid Gap:	("tid gap"). An achromatic (unstained) region in one chromatid, the size of which is equal to or smaller than the width of a chromatid. These are noted but not usually included in final totals of aberrations as they may not all be true breaks.
SG	Chromosome Gap:	("isochromatid gap, IG"). Same as chromatid gap but at the same locus in both sister chromatids.
UC	Uncoiled Chromosome:	Failure of chromatin packing. Probably not a true aberration.
PP	Polyploid cell:	A cell containing multiple copies of the haploid number (n) of chromosomes. Only indexed if very common. Not counted in the cells scored for aberrations.
E	Endoreduplication:	4n cell in which separation of chromosome pairs has failed. Only indexed if very common. Not counted in the cells scored for aberrations.

SIMPLE

TB	Chromatid Break:	An achromatic region in one chromatid, larger than the width of a chromatid. The associated fragment may be partially or completely displaced.
SB	Chromosome Break:	Chromosome has a clear break, forming an abnormal (deleted) chromosome with an acentric fragment (AF) that is dislocated. This classification now includes the AF. The AF was different from the SB only in that it was not apparently related to any specific chromosome.
DM	"Double Minute" fragment:	These are small double dots, some of which are terminal deletions and some interstitial deletions and probably small rings. Their origins are not distinguishable.

COMPLEX

ID		Interstitial Deletion: Length of chromatid "cut out" from midregion of a chromatid resulting in a small fragment or ring lying beside a shortened chromatid or a gap in the chromatid.
TR	Triradial:	An exchange between two chromosomes, or one chromosome and an acentric fragment, which results in a three-armed configuration.
QR	Quadriradial:	As triradial, but resulting in a four-armed configuration.

CR	Complex Rearrangement:	An exchange among more than two chromosomes or fragments which is the result of several breaks.
D	Dicentric:	An exchange between two chromosomes which results in a chromosome with two centromeres. This is often associated with an acentric fragment in which case it is classified as DF.
DF		Dicentric with fragment.
TC	Tricentric:	An exchange involving three chromosomes and resulting in a chromosome with three centromeres. Often associated with two to three AF. Such exchanges can involve many chromosomes and are named as follows:
QC	Quadricentric:	four centromeres, up to four AF
PC	Pentacentric:	five centromeres, up to five AF
HC	Hexacentric:	six centromeres, up to six AF
R	Ring:	A chromosome which forms a circle containing a centromere. This is often associated with an acentric fragment in which case it is classed as RF.
RC	Ring Chromatid:	Single chromatid ring (acentric).
RF		Ring with associated acentric fragment.
CI	Chromosome Intrachange:	Exchange within a chromosome; e.g., a ring that does not include the entire chromosome.
T	Translocation:	Obvious transfer of material between two chromosomes resulting in two abnormal chromosomes. When identifiable, scored as "T" not "2Ab."
AB		Abnormal monocentric chromosome. This is a chromosome whose morphology is abnormal for the karyotype, and often the result of a translocation, pericentric inversion, etc. Classification used if abnormality cannot be ascribed to; e.g., a reciprocal translocation.
OTHER		
GT/>		A cell which contains more than 10 aberrations. A heavily damaged cell should be analyzed to identify the types of aberrations and may not actually have >10, e.g., multiple fragments such as those found associated with a tricentric.

SECTION 6

MUTAGENICITY TEST ON CTFE TRIMER ACID IN THE *IN VIVO/IN VITRO* RAT PRIMARY HEPATOCYTE UNSCHEDULED DNA SYNTHESIS AND S-PHASE INDUCTION ASSAYS

M. A. Cifone

ABSTRACT

In the *In Vivo/In Vitro* Rat Primary Hepatocyte UDS assay, CTFE trimer acid did not induce significant changes in the nuclear labeling of rat hepatocytes over a dose range of 24.8 to 198 mg/kg. Three male F-344 rats were treated by oral gavage at each of four doses with the test material dissolved in corn oil. About 16.0 h after treatment with the test material, primary hepatocyte cultures were prepared. Viabilities of the hepatocytes obtained ranged from 83.3 to 93.8%. After attachment of the hepatocytes to the culture plates, the cultures were incubated with 10 μCi/mL ^{3}HTdr, (45 Ci/mmole) for 4 h. The cultures were prepared for analysis of nuclear labeling 16.4 h after removal of the radioactivity and addition of 0.25 mM unlabeled thymidine. None of the criteria used to indicate UDS were met and no dose-related response was observed. CTFE trimer acid was evaluated as inactive in the UDS assay. Although UDS activity was not observed at the 16-h timepoint, a significant elevation in S-phase DNA synthesis that met the criteria for a positive response was observed and addressed as part of the S-phase induction assay. The increase in replicative DNA synthesis indicated activity in the S-Phase Induction assay.

In the *In Vivo/In Vitro* Rat Primary Hepatocyte DNA Synthesis (S-Phase) Induction assay, CTFE trimer acid did not induce a significant increases in the number of S-phase cells over a dose range of 24.8 to 198 mg/kg. At least three male F-344 rats were treated by oral gavage at each of four doses with the test material dissolved in corn oil. About 48 h later, primary hepatocyte cultures were prepared for S-phase analysis. Viabilities of the hepatocytes prepared for S-phase analysis ranged from 76.3 to 96.2%. After attachment, the cultures were incubated with 10 μCi/mL ^{3}HTdr (45 Ci/mmole) for 4 h. The cultures were prepared for analysis of labeling 17.0 to 17.7 h after removal of the radioactivity and addition of 0.25 mM thymidine. While none of the treatments with the test article caused a significant elevation in the level of S-phase cells at the 48-h timepoint, a significant elevation in S-phase was observed in treated cultures at the 16-h timepoint. The elevation occurred in all three rats treated with 198 mg/kg and in two of three rats treated with either 99 mg/kg or 49.5 mg/kg. The test material was therefore evaluated as active in the DNA Synthesis (S-Phase) Induction Assay.

INTRODUCTION

These assays were designed to measure UDS or S-phase (replicative DNA synthesis) in rat liver cells using the techniques described by Williams (1980). Fresh hepatocytes obtained from rat liver will attach to a surface in culture and continued to metabolize for several days without undergoing cell division. Only a small percentage of the cells will enter S-phase. Therefore, if ^{3}HTdr is introduced into the culture medium, little or no label will be incorporated into nuclear DNA. Pretreatment of rats with a test material that interacts with the DNA will stimulate a repair response in which the altered portion of DNA is excised and the missing region replaced by DNA synthesis. This synthesis of DNA by nondividing cells is known as UDS and can be measured by determining the amount of ^{3}HTdr incorporated into DNA. This measurement of DNA repair appears to correlate very well with the known

mutagenic or carcinogenic activities of chemicals (Williams, 1980). The use of autoradiographic techniques affords simultaneous measurements of UDS and S-phase in hepatocyte cultures because S-phase cells are very heavily labeled and easily distinguished from lightly labeled cells exhibiting UDS. Hepatotoxicants such as carbon tetrachloride and dinitrotoluene induce an increase in cell proliferation to replace necrotic tissue (Butterworth et al., 1987; Mirsalis and Butterworth, 1982). Other compounds may induce S-phase synthesis in the absence of hepatotoxicity.

The objective of this assay was to detect DNA damage caused by the test material, or an active metabolite, by measuring UDS or S-phase induced in rat primary hepatocytes *in vivo*. The existence and degree of DNA damage was inferred from an increase in net nuclear grain (NNG) counts in hepatocytes obtained from treated animals when compared to those from untreated animals. The types of DNA damage are unspecified but must be recognizable by the cellular repair system and result in the incorporation of new bases (including ^{3}HTdr) into DNA during a short *in vitro* culture period. Quantitation of cells in S-phase has been shown to be useful for the evaluation of chemicals that may cause increased cell proliferation in the liver (Butterworth et al., 1987).

MATERIALS AND METHODS

Indicator Cells

The indicator cells for the UDS assay were hepatocytes obtained from adult male F-344 rats weighing from 180.5 to 199.6 g, purchased from Charles River Breeding Laboratories, Inc. The animals used for the S-phase assay were obtained from the same supplier and weighed from 180.0 to 203.9 g. The animals were fed Purina Certified^{R} Rodent Chow (Formula 5002) and water *ad libitum*. Animals were identified by ear tag after random assignment to the study and were quarantined a minimum of five calendar days prior to use.

Animals were anesthetized prior to surgery using 60 mg/kg pentobarbital and were exsanguinated during the liver perfusion. The cells were obtained by perfusion of the livers *in situ* with media containing type II collagenase. Monolayer cultures were established in culture dishes and were used the same day for analysis of the UDS or S-phase activity. All cultures were maintained as monolayers at 37°C in a humidified atmosphere containing approximately 5% CO_2.

Media

The cell cultures were established in Williams' Medium E supplemented with 10% FBS, 2 mM L-glutamine, 100 µg/mL streptomycin sulfate, and 150 µg/mL gentamycin (WME+). WME+ without serum is referred to as WMEI. After the establishment period, cultures were refed with WMEI containing 10 µCi/mL ^{3}HTdr, (WMEI-treat).

Controls

A vehicle control consisting of a minimum of three rats treated with corn oil by oral gavage was performed in all cases and at all timepoints. The dosing volumes did not exceed 2.03 mL/kg (a target of 2.0 mL/kg ± 10%).

The positive control article used is known to induce UDS *in vivo* in rat hepatocytes. DMN, delivered by ip injection at approximately 15 mg/kg was used for the UDS timepoint. For the S-phase timepoint, DMN was administered by ip injection at 20 mg/kg.

Test Article

CTFE trimer acid was obtained from the U.S. Air Force and was identified as ID# 10-86-63, IR# 14240. The clear, colorless, and slightly viscous liquid was stored in the dark in its original container.

Dosing Procedure

For both the UDS and S-phase assays, rats were treated by oral gavage with the test article solubilized in corn oil. The total volume of the test article solution administered did not exceed 2.03 mL/kg (target of 2.0 mL/kg ± 10%). DMN was solubilized in sterile deionized water and dosed as described previously. Fresh preparations of test article in vehicle were used for all testing purposes.

Dose Selection and Perfusion Time

For the UDS assay, the highest dose selected was 198 mg/kg. Three additional doses of test material were prepared using approximate 2-fold dilutions and a minimum of 3 animals per dose. The timepoint for harvesting hepatocytes for the UDS assay was 16.0 to 16.3 h after the administration of a single dose of the test article by oral gavage.

A dose of 198 mg/kg was selected as the maximum dose for the S-phase induction assay. Three additional doses of test material were prepared using approximate 2-fold dilutions and a minimum of 3 animals per dose. S-phase analysis was performed at 47.0 to 48.3 h after the administration of a single dose of the test article by oral gavage.

UDS and S-Phase Assays

These assays were based on the procedures described by Williams (1980), Mirsalis, Tyson, and Butterworth (1982), and Butterworth et al. (1987). The hepatocytes for both assays were obtained by perfusion of livers *in situ* for two to four min with Hanks' balanced salts (Ca^{++} - Mg^{++}-free) containing 0.5 mM ethyleneglycol-bis (ß-aminoethyl ether)-N, N-tetraacetic acid, and HEPES buffer at pH 7.2. Following this WMEI containing 50-100 U/mL of Type II collagenase was perfused through the liver for 10 to 11 min. The hepatocytes were obtained by mechanical dispersion of excised liver tissue in a culture dish containing the WMEI culture medium and collagenase. The suspended tissue and cells were allowed to settle to remove cell clumps and debris. The cell suspension was centrifuged and the cell pellet resuspended in WME+. After obtaining a viable cell count, a series of 35-mm culture dishes (at least 6 per animal containing a 25-mm round, plastic coverslip and at least 2 per animal to assess attachment efficiency) was inoculated with approximately 0.5×10^6 viable cells in 3 mL of WME+ per dish.

An attachment period of 1.5 to 2 h at 37°C in a humidified atmosphere containing 5% CO_2 was used to establish the cell cultures. Unattached cells were then removed and the cultures were refed with 2.5 mL WME-treat. Any remaining cultures were kept for analysis in the event of technical problems with autoradiography. Attachment efficiency was determined for two cultures from each animal using trypan blue dye exclusion and *in situ* analysis.

After a labeling period of 4 h labeled cultures were refed with WMEI containing 0.25 mM unlabeled thymidine. The cells were returned to the incubator for 16 to 20 h. After the incubation period, the hepatocyte nuclei were swollen by addition of 1% sodium citrate to the coverslips (containing the cell monolayers) for 10 min. The cells were fixed in acetic acid:ethanol (1:3) and dried for at least 24 h. The fixed coverslips were mounted on glass slides, dipped in Kodak NTB2 emulsion, and dried. The emulsion coated slides were stored for seven days (for UDS assay) or eight days (for S-Phase assay)

at 4°C in light-tight boxes containing Drierite. The emulsions were then developed in D19, fixed, and stained with Williams' modified hematoxylin and eosin procedure.

For the UDS assay the cells were examined microscopically at approximately 1500x magnification under oil immersion and the field was displayed on the video screen of an automatic counter. UDS was measured by counting nuclear grains and subtracting the average number of grains in three nuclear-sized areas adjacent to each nucleus (background count). This value is referred to as the NNG count. The coverslips were coded to prevent bias in grain counting.

The NNG count was determined for fifty randomly selected cells on each coverslip analyzed for UDS, unless otherwise indicated. The cells were counted by positioning the slide under the microscope in the upper right section of the slide and noting the vertical vernier. The first ten analyzable cells were counted moving the field to the left. After counting ten cells, the slide was moved vertically to another position and counting resumed. This process was repeated until fifty cells on the slide were counted. Only normally appearing nuclei were scored, and any occasional nuclei blackened by grains too numerous to count were excluded as cells in which replicative DNA synthesis occurred rather than repair synthesis. The mean NNG count was determined from triplicate coverslips (150 total nuclei) for each animal and from at least two animals per treatment condition.

Cells undergoing DNA replication were easily distinguished from nonreplicating cells in autoradiographic preparations. For the S-phase time point (48 h), the percentage of cells in S-phase (%S) was calculated manually as those cells exhibiting nuclei blackened by grains too numerous to count. At least 2000 cells were counted from randomly selected areas of each slide. Replicating and nonreplicating cells were counted separately using a two-position hand counter. For each dose, 6000 cells were scored for each of 3 animals (18,000 total cells per dose), unless indicated otherwise.

RESULTS AND DISCUSSION

UDS Assay

CTFE trimer acid was dissolved in corn oil at concentrations ranging from 12.7 to 102 mg/mL. All dosing stocks were individually prepared just prior to use. The test material appeared to form a clear, colorless solution in the vehicle. For the UDS assay, groups of three rats were each dosed with 198, 99.0, 49.4, or 24.8 mg/kg of test article in volumes that did not exceed 2.03 mL/kg.

Perfusions were initiated 16.0 to 16.3 h after administration of a single dose of the test article by oral gavage. The hepatocytes collected ranged in viability (determined by trypan blue exclusion) from 83.3 to 93.8% of the total cells collected in the perfusate (Table 6-1). The attachment efficiency varied from 46.1 to 88.5% and the viability of the attached cells was very good, ranging from 74.6 to 91.5%.

TABLE 6-1. SUMMARY OF CULTURE DATA FOR HEPATOCYTES FROM RATS[a] TREATED WITH CTFE TRIMER ACID (16-HOUR TIMEPOINT)

Target Dose (mg/kg)	Animal Number	Perfusion Viability (%)	Attachment[b] Efficiency (%)	Attachment[b] Viability (%)
VC[c]	3369	91.3	46.1	90.4
VC	3374	84.8	70.1	86.3
VC	3386	91.3	78.9	91.2
DMN[d]	3373	90.4	80.3	90.4
DMN	3375	91.7	72.2	91.5
DMN	3378	89.7	77.4	89.7
24.8	3380	89.4	60.8	88.2
24.8	3383	91.6	77.9	89.5
24.8	3384	93.8	64.5	88.2
49.5	3370	93.1	46.5	77.2
49.5	3376	88.9	50.2	76.5
49.5	3382	89.3	49.0	74.6
99.0	3371	83.3	69.1	87.0
99.0	3377	84.5	66.7	85.7
99.0	3381	85.6	61.1	80.5
198.0	3372	89.7	83.1	87.4
198.0	3379	89.0	83.1	86.8
198.0	3385	86.4	88.5	86.5

[a] Three animals per dose were treated.
[b] Results based on viable counts (trypan blue dye exclusion) of randomly selected areas on two plates.
[c] VC = Vehicle control (corn oil, 2 mL/kg).
[d] DMN = Dimethylnitrosamine (15 mg/kg).

The minimum criteria for a UDS response were determined by comparison with the average of the concurrent vehicle control treatments. A positive response consisted of mean NNG counts exceeding 4.40 (5 above the pooled average net grain count of -0.60) or at least 10.4% of the nuclei containing 5 or more grains (10% above the pooled average of 0.4%). None of the treatments with the test material samples caused nuclear labeling that was significantly different from the vehicle control (Table 6-2). Furthermore, no dose-related trend was evident. In contrast, the DMN treatments induced a large increase in nuclear labeling that greatly exceeded both criteria used to indicate UDS (See Appendix 6-A). Because the positive control animals were responsive to DMN, the test results provided conclusive evidence for the lack of UDS induction by the test material.

Heavily labeled nuclei (blackened with numerous grains) represent cells undergoing DNA replication as opposed to DNA repair. The number present in this study was low and did not interfere with the assay. Only 10 cells (0.22%) among the 4500 vehicle control cells screened in the entire assay were heavily labeled.

TABLE 6-2. UDS DATA SUMMARY FOR HEPATOCYTES FROM RATS[a] TREATED WITH CTFE TRIMER ACID (16-HOUR TIMEPOINT)

Target Dose (mg/kg)	Animal Number	Net Nuclear Grains ± SD[b]	Mean Cyto Grains[c]	% Nuclei with ≥5 Net Grains[c]	% S[d]
VC[e]	3369	-0.58 ± 0.06	5.53	0.0	0.13
VC	3374	-0.81 ± 0.23	5.60	0.0	0.27
VC	3386	-0.41 ± 0.54	5.54	1.3	0.27
DMN[f]	3373[g]	3.99 ± 1.60	6.94	41.3	0.60
DMN	3375[g]	9.22 ± 0.71	5.97	88.7	0.53
DMN	3378	5.43 ± 1.64	4.80	56.0	0.60
24.75	3380	-0.15 ± 0.48	6.00	0.7	0.53
24.75	3383	-0.76 ± 1.02	7.25	1.3	1.60
24.75	3384	-0.22 ± 0.07	5.75	2.0	0.87
49.5	3370[h]	---	--	--	--
49.5	3376	0.15 ± 0.58	5.99	5.3	3.87*
49.5	3382[g]	-0.72 ± 0.01	7.17	0.0	1.27*
99.0	3371	-0.67 ± 0.22	6.90	1.3	0.87
99.0	3377	-0.78 ± 0.34	7.10	4.0	2.00*
99.0	3381[g]	-0.57 ± 0.76	7.11	6.7	1.87
198.0	3372	0.59 ± 0.57	6.63	6.7	2.67*
198.0	3379	0.17 ± 0.45	5.74	4.7	1.60*
198.0	3385	0.51 ± 0.17	4.90	3.3	2.33*

[a] Three animals per dose were treated.
[b] = Average of NNG counts on triplicate coverslips (150 total cells), ± standard deviation between coverslips.
[c] Average values for triplicate coverslips.
[d] Percent of S-phase nuclei on triplicate coverslips (1500 total cells scored).
[e] VC = Vehicle control (corn oil, 2 mL/kg).
[f] DMN = Dimethylnitrosamine (15 mg/kg).
[g] One slide not analyzable; averages based upon analysis of duplicate coverslips (150 total cells).
[h] No slides suitable for analysis.
* = Statistically significant difference from pooled vehicle control values, $p < 0.05$.

S-Phase Assay

In order to determine the amount of cell proliferation induced by the test material, groups of three rats were dosed with the test material at 24.8, 49.5, 99, or 198 mg/kg and sacrificed about 48 h after a single dose of the chemical was administered.

The hepatocytes collected for S-phase analysis ranged in viability (determined by trypan blue exclusion) from 76.3 to 96.2% of the total cells collected in the perfusate (Table 6-3). The attachment efficiency varied from 58.1 to 94.5% and the viability of the attached cells was very good, ranging from 77.4 to 94.3%.

TABLE 6-3. CULTURE DATA SUMMARY FOR HEPATOCYTES FROM RATS[a] TREATED WITH CTFE TRIMER ACID (48-HOUR TIMEPOINT)

Target Dose (mg/kg)	Animal Number	Perfusion Viability (%)	Attachment[b] Efficiency (%)	Attachment[b] Viability (%)
VC[c]	3407	90.0	83.1	87.4
VC	3411	91.7	81.8	85.2
VC	3413	91.6	84.6	85.8
DMN[d]	3415	87.3	75.2	91.0
DMN	3416	91.0	79.2	94.3
DMN	3418	89.4	94.5	91.3
24.8	3412	96.2	74.4	94.1
24.8	3417	93.2	62.9	77.4
24.8	3419	85.1	94.5	92.0
49.5	3405	85.6	66.7	90.8
49.5	3421	94.1	77.2	90.6
49.5	3422	87.4	73.0	86.4
99.0	3409	88.3	64.4	86.9
99.0	3410	76.3	58.1	86.1
99.0	3414	93.5	72.0	90.8
198.0	3406	88.7	67.1	89.3
198.0	3408	84.3	62.1	89.4
198.0	3420	88.0	74.2	88.2

[a] Three animals per dose were treated.
[b] Results based on viable counts (trypan blue dye exclusion) of randomly selected areas on two plates.
[c] VC = Vehicle control (corn oil, 2 mL/kg).
[d] DMN = Dimethylnitrosamine (20 mg/kg).

No significant increase in DNA synthesis was observed in the treated animals when compared to the average of the pooled vehicle control treatments (Table 6-4). In contrast, a significant elevation of S-phase DNA synthesis was observed in cells obtained from the positive control treatments. The test article was considered negative for replicative DNA synthesis at the 48-h timepoint.

TABLE 6-4. S-PHASE DATA FOR HEPATOCYTES FROM RATS[a] TREATED WITH CTFE TRIMER ACID

Target Dose (mg/kg)	Animal Number	Total[b] S-Phase	Total[b] Cells Analyzed	% S-Phase ± SD
VC[c]	3413	32	6000	0.53 ± 0.13
VC	3407	75	6000	1.25 ± 0.49
VC	3411	59	6000	0.98 ± 0.31
DMN[d]	3416[e]	519	6000	8.65 ± 0.88
DMN	3415	348	6000	5.80 ± 2.23
DMN	3418	333	6000	5.55 ± 0.53
24.8	3412	33	6000	0.55 ± 0.38
24.8	3417	15	6000	0.25 ± 0.18
24.8	3419	34	6000	0.57 ± 0.13
49.5	3421	117	6000	1.95 ± 0.33*
49.5	3405	31	6000	0.52 ± 0.13
49.5	3422	71	6000	1.18 ± 0.24
99.0	3409	13	6000	0.22 ± 0.10
99.0	3410	25	6000	0.42 ± 0.25
99.0	3414	58	6000	0.97 ± 0.21
198.0	3408	9	6000	0.15 ± 0.09
198.0	3420	17	6000	0.28 ± 0.14
198.0	3406	38	6000	0.63 ± 0.41

[a] Three animals per dose were treated.
[b] Triplicate coverslips, 2000 cells/coverslips.
[c] VC = Vehicle control (2 mL/kg of corn oil).
[d] DMN = Dimethylnitrosamine, 20 mg/kg.
[e] Two coverslips analyzed, 3000 cells/coverslip.
* = Statistically significant difference compared to the average of the pooled vehicle controls $p < 0.05$.

A significant elevation in DNA synthesis was observed in treated animals sacrificed at the 16-h timepoint (Table 6-2). During UDS analysis, 500 cells per slide (1500 cells per animal) were analyzed for S-phase. The elevation in S-phase was significant in comparison to the pooled vehicle control value in all animals receiving 198 mg/kg, in one of three animals dosed with 99.0 mg/kg, and in two of three animals dosed with 49.5 mg/kg of test article. A single animal dosed with 99.0 mg/kg demonstrated an S-phase response that exceeded 1% but was not considered significant because of the variability between slides. S-phase response at this time period may indicate early induction of toxicity leading to a proliferative response or may be due to a mitogenic stimulus. The results provide evidence of induction of S-phase by CTFE trimer acid at the 16-h timepoint.

CONCLUSIONS

The test material, CTFE trimer acid, did not induce significant changes in the nuclear labeling of rat primary hepatocytes for a dose range of 24.8 to 198 mg/kg. CTFE trimer acid was therefore evaluated as inactive in the *In Vivo/In Vitro* Rat Primary Hepatocyte UDS assay.

No S-phase response was observed for the test material over a dosing range of 24.8 to 198 mg/kg at the 48-h timepoint. A significant S-phase response was observed for the test material over a dosing range of 49.5 to 198 mg/kg at the 16-h timepoint. CTFE trimer acid was therefore evaluated as active in the *In Vivo/In Vitro* Rat Primary Hepatocyte S-Phase Induction assay.

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APPENDIX 6-A

ASSAY ACCEPTANCE AND EVALUATION CRITERIA

Assay Acceptance Criteria

An assay normally will be considered acceptable for evaluation of the test results only if all of the criteria listed below are satisfied. This listing may not encompass all test situations, so scientific judgment must be exercised in modifying the criteria or considering other causes that might affect assay reliability and acceptance.

1. The viability of the hepatocytes collected from the perfusion process normally exceeds 70%. A variety of factors can affect cell yield and viability, so values below 70% are not uncommon nor necessarily detrimental. Toxicity of treatment with test article may be reflected in perfusion viability, therefore no lower limit will be set.

2. The viability of the monolayer cell cultures used for the assay must be 70% or greater. Normally, the viability of attached cells is about 85%.

3. The positive control is used to demonstrate that the cell population employed was responsive and the methodology was adequate for the detection of UDS or S-phase. For test materials causing weak or no UDS activity, the average response to the positive control treatments must exceed either criteria used to indicate UDS. The positive control for S-phase must have greater than 1% of the cells in scheduled DNA synthesis. For test materials clearly causing a dose-related UDS activity or S-phase activity, an assay will be acceptable in the absence of a positive control lost for technical reasons. Historical values for the UDS positive control are NNG = 8.79 ± 3.92 (range, 1.55 to 15.40) and % ≥ 5 grains per nucleus = 62.7% ± 18.2% (range 20.7% to 89.7%). Historical control values for the UDS negative control are NNG = -0.54 ± 0.50 (range, -1.74 to 0.12) and % ≥ 5 grains per nucleus = 1.4% ± 1.7% (range 0.0% to 6.7%).

4. Grain count data obtained per animal is acceptable as part of the evaluation if obtained from two replicate cultures and at least 50 nuclei per culture for UDS or 2000 cells per culture for S-phase. Data should be available from 2 of the 3 animals treated.

5. A minimum of three doses will be analyzed for nuclear grain counts. Repeat trials need only augment the number of analyzed doses in the first trial to achieve a total of 3 concentrations, but must include one dose previously analyzed as acceptable.

Assay Evaluation Criteria

UDS Assay

Several criteria have been established which, if met, provide a basis for evaluation of a test material as active in the UDS assay. The criteria for a positive response are based on a statistical analysis of the historical control data as described by Casciano and Gaylor (1983). The test material is considered active in the UDS assay at doses that cause the following.

1. An increase in the mean NNG count to at least five grains per nucleus above the concurrent vehicle control value leading to a positive number, and/or;

2. An increase in the number of nuclei having five or more net grains such that the percentage of these nuclei in test cultures is 10% above the concurrent vehicle control cultures.

Generally, if the first condition is satisfied, the second will also be met. However, satisfaction of only the second condition can also indicate UDS activity. Different DNA-damaging agents can give a variety of nuclear labeling patterns, and weak agents may strongly affect only a minority of the cells. Therefore, both of the above conditions are considered in an evaluation. In cases where increases are not observed in all three animals, the test material will be considered active for that condition if cells from two of the three animals show increases. If the negative control shows an average less than -5 or more than one grain per nucleus, the assay will normally be considered invalid.

The test material is considered inactive in this assay if the following is true.

1. The mean NNG counts for all dosed groups is < 1.0 net nuclear grain count above the concurrent vehicle control value and/or;

2. The percent of nuclei with five or more net grains does not increase more than 2% above the concurrent vehicle control.

When results are neither clearly positive nor clearly negative, the presence of a dose response, the frequency distribution of cellular responses, and the reproducibility of data among animals is considered; the test article is then classified as "negative", "weak positive", or "equivocal". Groups in which one of three animals shows increases in labeling will be decided on a case-by-case basis depending on the level of activity in cells from the active animal, the level of activity in cells from the inactive animals and the presence or absence of activity in surrounding groups.

The positive control nuclear labeling is not used as a reference point to estimate mutagenic or carcinogenic risk associated with the UDS activity of the test material. UDS elicited by test agents in this assay is probably more dependent on the type of DNA damage inflicted and the available repair mechanisms than on the potency of the test agent as a mutagen or carcinogen. Some forms of DNA damage are repaired without the incorporation of new nucleic acids. Thus, the positive controls are used to demonstrate that the cell population employed was responsive and the methodology was adequate for the detection of UDS.

S-Phase Induction Assay

The test article will be considered positive in the S-phase induction assay if the % S in cells from all animals in a group is significantly greater than the pooled vehicle control and exceeds 1% of the population. Significance will be determined using Student's t-test for unpaired observations modified for unequal variances when an F-test performed on the data indicates that the variances were unequal. A standard t-test will be used when the variances are equal.

The test article will be considered negative if the % S for all treatment groups is < 1% of the population (historical negative controls have < 1% of the cells undergoing DNA replication). The data base for S-phase is not extensive and some judgement may be needed in the evaluation of a response.

SECTION 7

IN VITRO TRANSFORMATION OF BALB/c-3T3 CELLS WITH AND WITHOUT S9 ACTIVATION OF CTFE TRIMER ACID

B.C. Myhr

ABSTRACT

CTFE trimer acid was assayed for its ability to induce morphological cell transformation in BALB/c-3T3 cell cultures in the absence and presence of a rat liver S9 metabolic activation system. Two independent assays were performed under each condition. The treatment period was 72 h without activation and 4 h in the presence of S9 metabolic activation. In the absence of an S9 metabolic activation system, six to seven treatments ranging from 100 to 800 μg/mL were examined in two trials. The toxicity, determined from the clonal survival of ouabain-resistant cells in the presence of a wildtype monolayer, ranged from 10.8 to 96.0%. The number of transformed foci in the CTFE trimer acid-treated cultures did not change significantly from the corresponding negative controls. In the presence of S9 metabolic activation, a shift in toxicity was observed and six treatments from 500 to 2000 μg/mL were analyzed in each trial. Again a wide range of toxicities was induced (5.6 to 100%) and a dose-related significant increase in the number of transformed foci was not observed in the CTFE trimer acid-treated cultures. CTFE trimer acid was therefore evaluated as negative for the induction of morphological transformation in BALB/c-3T3 cell cultures.

INTRODUCTION

BALB/c-3T3 mouse cells multiply in culture until a uniform monolayer is achieved and then cease further division (Kakunaga, 1973; Rundell, 1984b). These nontransformed cells, if injected into immunosuppressed mice (1×10^7 cells/animal), do not produce neoplastic tumors (Kakunaga, 1973; Rundell et al., 1983; Rundell, 1984a). However, BALB/c-3T3 cells treated *in vitro* with some chemical carcinogens give rise to foci of morphologically altered cells superimposed on the contact-inhibited cell monolayer. If foci picked from cell cultures are grown to larger cell numbers and are injected into immunosuppressed mice, a malignant tumor will be obtained in most cases. Thus, the appearance of foci of altered cells is correlated with malignant transformation.

The ability of BALB/c-3T3 cells to metabolize test articles from various chemical classes can be enhanced by the addition of an exogenous S9 metabolic activation system to the cultures during the treatment period. However, the standard treatment period of 72 h is reduced to only 4 h because of S9 toxicity and the degradation of the NADPH-dependent microsomal enzyme system, so this assay modification may not always detect procarcinogens. The procarcinogen, DMN, does not transform BALB/c-3T3 cells in the absence of S9 (Rundell et al., 1983), but DMN treatments with the S9 activation system usually induce a statistically significant increase in the frequency of transformed foci (Matthews and Rundell). Similarly, S9-dependent induction of transformed foci by DMN has been reported for another mouse line (Tu et al., 1984).

An appropriate dose range for toxic test articles is selected by a novel method for determining clonal survival under the mass culture conditions of the transformation assay. Normal BALB/c-3T3 cells are sensitive to the cell membrane poison, ouabain, and are quickly killed. However, a mutant BALB/c-3T3 cell line has been established that is ouabain-resistant but otherwise as sensitive to test articles as the parent (wildtype,

ouabain-sensitive cells). When a few (eg., 600) ouabain-resistant cells are mixed with a large number of wildtype cells (eg., 7 x 10^4 cells), the clonal survival of the ouabain-resistant cells can be determined by the addition of 4 mM ouabain to the culture medium after the treatment period. In this manner, the test article toxicity is determined under the same cellular exposure conditions that will occur in the transformation assay mass cultures.

The objective of this assay was to evaluate CTFE trimer acid for its ability to induce the malignant transformation of cultured BALB/c-3T3 mouse cells in the absence and presence of a rat liver S9 metabolic activation system. Transformation has been defined as a dense, piling up of morphologically altered cells, called a focus, superimposed on a monolayer of contact-inhibited cells (Heidelberger et al., 1983; Rundell, 1984a;b).

MATERIALS AND METHODS

Indicator Cells

Clone 1-13 of BALB/c-3T3 mouse cells was obtained (Kakunaga, 1973). A subclone of these cells with a low spontaneous frequency of transformants was used for this study. Stocks of cells were maintained in liquid nitrogen and were checked to ensure the absence of *Mycoplasma* contamination. Laboratory cell cultures were grown in Eagle's minimum essential medium (EMEM) supplemented with FBS, L-glutamine, and antibiotics.

Controls

The negative control (also referred to as the solvent control) was EMEM culture medium containing 0.25% v/v DMSO. In addition, for the S9 activation assay, the S9 activation components were added to the medium.

A known carcinogen, MCA, was used at a concentration of 2.5 μg/mL as a positive control for the transformation of BALB/c-3T3 cells in the absence of S9. A second known carcinogen, DMN, was used as the positive control for the transformation of BALB/c-3T3 cells in the presence of S9 activation. DMN requires activation by S9 microsomal enzymes to become transforming, and two concentrations of DMN (1 and 2 μL/mL) were chosen to demonstrate a significant response for at least one concentration of DMN.

Test Material

The test material, CTFE trimer acid, was a clear, colorless liquid. It was stored at room temperature in its original glass container in a chemical cabinet.

S9 Metabolic Activation System

A 9000 x g supernatant fraction (Ames et al., 1984) prepared from the liver of Aroclor 1254-induced Sprague-Dawley male rats was purchased from Molecular Toxicology, Inc. The S9 was prescreened and selected for relatively low toxicity to BALB/c-3T3 cells and for the conversion of DMN to toxic metabolites. The concentration of S9 selected for the assay was 40 μL/mL that corresponded to 1.5 mg S9 protein/mL in the treatment medium.

The S9 activation components included a NADPH regenerating system composed of NADP and isocitric acid in the presence of S9. The final concentration of the components in the treatment medium was approximately 236 μg/mL of NADP (sodium salt) and approximately 1552 μg/mL of isocitrate. The regenerating system solution was prepared fresh as a 4X stock solution in culture medium and was combined with an equal volume of diluted S9 to give a 2X S9 mix. The mix was held on ice until used in the assay.

Test Material Preparation

CTFE trimer acid was soluble in DMSO up to a concentration of 2000 mg/mL. The primary stocks for the cytotoxicity and transformation assays were diluted with EMEM containing 10%v/v FBS (EMEM10) to prepare 2X dosing solutions and further dilutions were prepared in EMEM(10) containing 0.5% DMSO. The primary medium stock for the transformation assays (10,000 μg/mL) was cloudy and yellow with a precipitate that settled with time. These stocks were thoroughly mixed prior further dilution. The cultures were dosed by adding 4 mL of the appropriate dosing medium to 4 mL of EMEM(10) already present in the culture flasks. The test material appeared to be soluble in the dosed cultures but treatments at and above concentration of 2000 μg/mL were yellow. The yellow coloration of the culture media did not appear to be caused by a change in pH of the media.

Preliminary Dose Rangefinding

Glass culture bottles with approximately 60 cm^2 of surface area were seeded concurrently with approximately 600 ouabain-resistant 3T3 cells and 7×10^4 wildtype cells. The following day, one culture was exposed to each of 10 doses with and without S9, starting at 1000 μg/mL and diluting in 2-fold steps. Two solvent control cultures containing 0.25% DMSO in EMEM(10) culture medium were prepared for both test conditions. After an exposure period of approximately 4 h in the presence of the S9 activation system or approximately 72 h without S9, the cells were washed with a physiological solution and refed with EMEM(10) culture medium containing 4 mM ouabain. The cultures were refed with medium containing 4 mM ouabain 4 to 5 days later. Surviving colonies were terminated 9-11 days after the treatment period, stained with Giemsa, and counted manually.

A relative survival for each treatment condition was obtained by comparing the number of surviving colonies to the average colony count for the solvent control cultures. This survival information was used to select doses for the transformation assay that would span an anticipated survival range of 10 to 100%.

Transformation Assay

The transformation assay procedure was adapted from that reported by Kakunaga (1973). Glass culture bottles having a monolayer growth area of approximately 60 cm^2 were used. Each bottle was seeded with approximately 7×10^4 cells for tne transformation assay and 7×10^4 cells plus approximately 600 ouabain-resistant cells for the concurrent clonal survival assay. On the day after seeding, nine cultures were exposed to each selected treatment with CTFE trimer acid and the positive controls. Eighteen cultures were used as solvent controls for each of the test conditions (with and without the S9 activation system). One mixed culture of wildtype and ouabain-resistant cells was exposed to each treatment and positive control condition; two mixed cultures were included for the solvent controls.

The treatments were conducted at 37 ± 1°C for approximately 4 h with the S9 activation system and approximately 72 h without S9. The treatments were initiated by adding 4 mL of a 2X stock of CTFE trimer acid or solution of the positive control to each culture containing 4 mL of culture medium (with or without the S9 activation system). After treatment, all cultures were washed with Hanks' balanced salt solution. The transformation assay cultures were refed with fresh EMEM culture medium, and the incubation was continued for 28 to 34 days with refeeding twice a week. The activation studies were incubated slightly longer to allow adequate expression of the transformed colonies. The

clonal survival cultures were refed with medium containing 4 mM ouabain. The cultures were refed with 4 mM ouabain 4 days later if needed and were terminated when the colonies reached a size that could be easily scored.

The cultures were terminated by fixing the cells with methanol and staining with 10% Giemsa in tap water. Stained cultures were examined by eye and by microscope to determine the number of foci of transformed cells and the colony survival. The transformation assay cultures were coded with random numbers prior to evaluation for foci.

Evaluation of Transformed Foci

At the end of the incubation period, cultures of cells with a normal phenotype yielded a uniformly stained monolayer of round, contact-inhibited cells. Transformed cells formed a multi-layered mass of cells, or focus, that stained deeply and was superimposed on the surrounding monolayer of cells (Kakunaga, 1973; Rundell et al., 1983; Rundell 1984a). The foci were variable in size and exhibited several variations in morphological features. Many scored foci consisted of a dense piling-up of cells with a random, criss-cross orientation of fibroblastic cells at the periphery of the focus and extensive invasiveness into the contiguous monolayer. Other scored foci were composed of more rounded cells with little criss-crossing at the periphery but with necrosis at the center caused by the dense piling-up of a large number of cells. A third variation was a focus without the necrotic center and large number of cells but which exhibited the criss-cross pattern of overlapping cells throughout most of the colony.

Some densely stained areas were not scored as transformed foci because the random orientation of fibroblastic cells was not observed. Microscopic examination is routinely employed for scoring and for the final judgement of the transformed character of each focus.

All foci that exhibited the transformed characteristics were scored. In the raw data, a record of focus size was maintained by scoring foci greater than 4 mm in diameter as +++ and those of 2 to 4 mm in diameter as ++. No significance is currently attached to this categorization. The sum of all scored foci (+++ and ++) was reported for each culture and was used for the assay analysis.

RESULTS AND DISCUSSION

Clonal Survival

CTFE trimer acid was lethal to BALB/c-3T3 cells at a concentration of 1000 μg/mL (Table 7-1). Moderate toxicity was induced at a concentration of 500 μg/mL (55.1% relative survival) and lower concentrations were essentially nontoxic. The addition of the rat liver S9 metabolic activation system (Table 7-2) caused a slight shift in toxicity. The test material was nontoxic at concentrations of 1000 μg/mL. These results were used to select doses for the transformation assays and results from the early transformation assays were used to further define the dose range. For the nonactivation studies used in the evaluation, treatments ranging from from 100 to 800 μg/mL were analyzed. In the presence of S9 metabolic activation, treatments from 500 to 2000 μg/mL were initiated.

TABLE 7-1. CYTOTOXIC ACTIVITY OF CTFE TRIMER ACID IN THE PRELIMINARY CLONAL SURVIVAL ASSAY WITHOUT S9 ACTIVATION

Treatment Condition	Colonies/Culture	Relative Cell Survival (%)
Solvent Control[a]	235, 262	100.0
CTFE Trimer Acid (μg/mL)		
1.00	223	89.7
2.50	222	89.3
5.00	230	92.6
10.0	226	90.9
25.0	219	88.1
50.0	237	95.4
100	232	93.4
250	236	95.0
500	137	55.1
1000	0	0.0

[a] EMEM culture medium containing 0.25% DMSO.

TABLE 7-2. CYTOTOXIC ACTIVITY OF CTFE TRIMER ACID IN THE PRELIMINARY CLONAL SURVIVAL ASSAY WITH S9 ACTIVATION

Treatment Condition	Colonies/Culture	Relative Cell Survival (%)
Solvent Control[a]	81, 97	100.0
CTFE Trimer Acid (μg/mL)		
1.00	101	113.5
2.50	74	83.1
5.00	78	87.6
10.0	66	74.2
25.0	91	102.2
50.0	102	114.6
100	100	112.4
250	96	107.9
500	108	121.3
1000	86	96.6

[a] EMEM culture medium containing 0.25% DMSO.

Preliminary Transformation Studies

Three transformation assays conducted in the absence and presence of S9 metabolic activation were initiated, but the first trial was subsequently terminated without the collection of any valid data due to contamination. At the same time, other transformation assays performed in the laboratory were terminated due to the observation of numerous transformed foci in the negative control cultures. This result caused the temporary interruption of all

studies until the reason(s) could be identified and eliminated. After several months, a new lot of FBS was identified that afforded normal behavior of the negative control cultures. This serum and a fresh batch of cells from the cryopreserved stock were used to reinitiate the study. The first trial with the new lot of serum and stock of cells was considered Trial 1 that was followed by Trial 2.

Transformation Assay Without S9

Table 7-3 summarizes the results obtained for CTFE trimer acid in the first complete trial of the transformation assay without S9. In Trial 1, six treatments ranging from 100 to 700 μg/mL were initiated and a wide range of toxicities was induced (46.6 to 96.0% relative survival). The frequency of transformed foci in the cultures exposed to CTFE trimer acid was not significantly elevated above the solvent control values. In contrast, the MCA positive control induced a significant increase in focus formation. In order to confirm the lack of activity by CTFE trimer acid, a second nonactivation assay was initiated.

TABLE 7-3. TRANSFORMING ACTIVITY OF CTFE TRIMER ACID ASSESSED IN THE TRANSFORMATION ASSAY USING BALB/c-3T3 CELLS WITHOUT S9 ACTIVATION TRIAL 1

Treatment Condition	Colony Count[a]	Relative Survival[b] (%)	Focus Data			Transforming Activity[c]
			Total Cultures	Total Foci	Average Foci/ Culture	Mean Foci/ Culture
Solvent Control[d]	253	100.0	18	1	0.06	0.04
Positive Control[e]	179	70.8	9	77	8.56	8.31**
CTFE Trimer Acid (μg/mL)						
100	236	93.3	9	0	0.00	0.00
200	238	94.1	9	1	0.11	0.08
400	243	96.0	9	1	0.11	0.08
500	206	81.4	9	0	0.00	0.00
600	164	64.8	9	3	0.33	0.26
700	118	46.6	9	1	0.11	0.08

[a] Clonal survival assay performed concurrently with the transformation assay.
[b] Colony survival relative to the solvent control.
[c] The mean transforming activity is expressed as the anti-log of the $\log_{10}$ mean minus one.
[d] Solvent Control: EMEM culture medium containing 0.25% DMSO.
[e] Positive Control: The positive control treatment was 3-MCA (2.5 μg/mL).
** $p < 0.01$.

Results from the second complete trial without S9 are shown in Table 7-4. Treatments ranging from 200 to 800 μg/mL were initiated in Trial 2. The 800 μg/mL treatment was added in an attempt to obtain higher toxicities. Survivals ranged from 10.8 to 93.1% of the solvent control value. An increase

in transformed foci was observed at concentrations of 200 μg/mL and 600 μg/mL and these increases were significant at the 95% confidence level. However, no dose-related trend was observed. The MCA positive control induced a large, significant increase in transformed foci. Because no significant dose-dependent increase in transformed foci was induced by CTFE trimer acid in two trials of the BALB/c-3T3 transformation assay, the test material was evaluated as nontransforming in the absence of S9 metabolic activation.

TABLE 7-4. TRANSFORMING ACTIVITY OF CTFE TRIMER ACID ASSESSED IN THE TRANSFORMATION ASSAY USING BALB/c-3T3 CELLS WITHOUT S9 ACTIVATION TRIAL 2

Treatment Condition	Colony Count[a]	Relative Survival[b] (%)	Focus Data			Transforming[c] Activity
			Total Cultures	Total Foci	Average Foci/ Culture	Mean Foci/ Culture
Solvent Control[d]	295.5	100.0	18	24	1.33	1.08
Positive Control[e]	236	79.9	9	51	5.67	5.30**
CTFE Trimer Acid (μg/mL)						
200	272	92.0	9	23	2.56	2.25*
400	275	93.1	9	17	1.89	1.47
500	274	92.7	9	12	1.33	0.85
600	219	74.1	9	19	2.11	2.02*
700	128	43.3	9	9	1.00	0.74
800	32	10.8	9	10	1.11	1.03

[a] Clonal survival assay performed concurrently with the transformation assay.
[b] Colony survival relative to the solvent control.
[c] The mean transforming activity is expressed as the anti-log of the $\log_{10}$ mean minus one.
[d] Solvent Control: EMEM culture medium containing 0.25% DMSO.
[e] Positive Control: The positive control treatment was 3-MCA (2.5 μg/ml).
* $p < 0.05$.
** $p < 0.01$.

Transformation Assay with S9

The results obtained in the first trial of the transformation assay with S9 are summarized in Table 7-5. In Trial 1, six treatments ranging from 500 to 2000 μg/mL were initiated and a good range of toxicities was induced (6.2 to 107.6% survival). No significant increase in the number of transformed foci was observed in any of the cultures exposed to CTFE trimer acid. Both of the DMN positive controls clearly induced foci. A second assay was initiated in the presence of S9 metabolic activation to confirm the lack of activity by the test material.

Table 7-6 summarizes the results obtained in Trial 2. The results from Trial 2 confirm the absence of activity by CTFE trimer acid in the presence of S9 metabolic activation. Six treatments ranging from 500 to 2000 μg/mL were

initiated and a wide range of toxicities was induced (5.6 to 91.6% relative survival). A small but significant increase in the number of transformed foci was observed at concentrations of 500 μg/mL and 1000 μg/mL. However, higher, more toxic treatments did not produce transformed foci. Both the DMN positive control treatments induced a large increase in transformed foci that was significant at the 99% confidence level. CTFE trimer acid was therefore evaluated as inactive in this assay in the presence of S9 metabolic activation.

TABLE 7-5. TRANSFORMING ACTIVITY OF CTFE TRIMER ACID ASSESSED IN THE TRANSFORMATION ASSAY USING BALB/c-3T3 CELLS WITH S9 ACTIVATION TRIAL 1

Treatment Condition	Colony Count[a]	Relative Survival[b] (%)	Focus Data			Transforming[c] Activity
			Total Cultures	Total Foci	Average Foci/ Culture	Mean Foci/ Culture
Solvent Control[d]	176.5	100.0	18	22	1.22	1.04
Positive Control[e]						
DMN, 1 μL/mL	171	96.9	9	32	3.56	3.03**
DMN, 2 μL/mL	101	57.2	9	19	2.11	1.96*
CTFE Trimer Acid (μg/mL)						
500	189	107.1	6[f]	0	0.00	0.00
1000	190	107.6	8[f]	7	0.88	0.62
1250	130	73.7	8[f]	1	0.13	0.09
1500	58	32.9	6[f]	5	0.83	0.78
1750	37	21.0	9	16	1.78	1.36
2000	11	6.2	9	25	2.78	1.67

[a] Clonal survival assay performed concurrently with the transformation assay.
[b] Colony survival relative to the solvent control.
[c] The mean transforming activity is expressed as the anti-log of the $\log_{10}$ mean minus one.
[d] Solvent Control: EMEM culture medium containing 0.25% DMSO.
[e] Positive Control: The positive control treatment was DMN at the concentrations shown.
[f] Contaminated cultures excluded.
* $p < 0.05$.
** $p < 0.01$.

TABLE 7-6. TRANSFORMING ACTIVITY OF CTFE TRIMER ACID ASSESSED IN THE TRANSFORMATION ASSAY USING BALB/c-3T3 CELLS WITH S9 ACTIVATION
TRIAL 2

Treatment Condition	Colony Count[a]	Relative Survival[b] (%)	Focus Data			Transforming[c] Activity
			Total Cultures	Total Foci	Average Foci/ Culture	Mean Foci/ Culture
Solvent Control[d]	231.5	100.0	18	10	0.56	0.40
Positive Control[e]						
DMN, 1 μL/mL	227	98.1	9	52	5.78	4.61**
DMN, 2 μL/mL	68	29.4	9	32	3.56	2.68**
CTFE Trimer Acid (μg/mL)						
500	200	86.4	9	13	1.44	1.19*
1000	212	91.6	9	19	2.11	1.77**
1250	176	76.0	8[f]	15	1.88	1.37
1500	82	35.4	8[g]	15	1.88	1.33
1750	32	13.8	9	9	1.00	0.88
2000	13	5.6	9	8	0.89	0.54

[a] Clonal survival assay performed concurrently with the transformation assay.
[b] Colony survival relative to the solvent control.
[c] The mean transforming activity is expressed as the anti-log of the $\log_{10}$ mean minus one.
[d] Solvent Control: EMEM culture medium containing 0.25% DMSO.
[e] Positive Control: The positive control treatment was DMN at the concentrations shown.
[f] Contaminated culture excluded.
[g] One culture not scored due to incomplete monolayer.
* $p < 0.05$.
** $p < 0.01$.

CONCLUSION

CTFE trimer acid was evaluated in the presence and absence of S9 metabolic activation within a dose range of 100 to 2000 μg/mL. Two independent trials were evaluated under each activation condition. A wide range of toxic responses was obtained, but no significant dose-dependent changes in the frequency of transformed foci were observed in the absence or presence of S9 metabolic activation. CTFE trimer acid was therefore evaluated as negative for transforming BALB/c-3T3 cells in culture.

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APPENDIX 7-A

ASSAY ACCEPTANCE AND EVALUATION CRITERIA

Assay Acceptance Criteria

The clonal survival assay conducted simultaneously with the transformation assay was considered acceptable for evaluation of the test results by meeting the following three criteria.

1. The negative (solvent) control cultures were macroscopically visible BALB/c-3T3 cell colonies representing a cloning efficiency of 15% or greater.

2. At least one of the test material treatments resulted in 10% to 50% cell survival.

3. A cytotoxic dose response was obtained for the test material treatments, unless the test material was nontoxic at 10 mg/mL or its solubility limit in culture medium was exceeded.

The transformation assay was considered acceptable for evaluation of test results by meeting the following five criteria.

1. Negative (solvent) control, positive control, and test material treatments resulted in contiguous monolayers of cells to be evaluated.

2. Negative control spontaneous frequencies of transformation did not exceed an average of approximately two foci per culture.

3. At least one of the positive control treatments resulted in an average number of foci per culture vessel that was significantly different from the negative control at the 99% confidence level ($p \leq 0.01$).

4. A minimum number of six culture vessels per test condition was available for analysis.

5. A minimum number of three treatment levels of the test material was available for analysis.

In addition, the cytotoxicity dose-related data from the preliminary and simultaneous clonal survival assays were qualitatively similar over a comparable range of test chemical treatments.

Assay Evaluation Criteria

The appearance of transformed foci usually occurs as a general increase in foci for all cultures exposed to a transforming dose. However, large numbers of foci may appear at random in one or more culture vessels in a treatment set, resulting in skewing of the mean number of foci in that set. This skewing could be caused by factors such as mechanical disruption and respreading of transformed foci cells or a culture-conditioning effect caused by the early appearance of a focus. The appearance of occasional dishes with numerous foci is a random process and occurs in both treated and control cell cultures. In our laboratory, we have utilized a $\log_{10}$ mathematical transformation to handle this non-normal distribution of BALB/c-3T3 cell

transformed foci data (Rundell et al., 1983); however, other mathematical models have also been proposed (Whorton et al., 1982). After performing a $\log_{10}$ transformation of the data, Bailey's modification of the Student's t-test (Bailey, 1959) was used to evaluate positive control and test chemical treatment transforming activity for significant differences from the negative control. The possible spectrum of responses was routinely subdivided into three levels for the evaluation of each treatment.

Evaluation of Individual Treatments

Strong positive response	=	$p \leq 0.01$
Weak positive response	=	$p \leq 0.05$
Negative response	=	$p > 0.05$

The results of each treatment condition were evaluated in relation to the observed activities of model compounds, and scientific judgement was exercised in the evaluation of each test material.

In general, a response at only one dose attaining a 95% confidence level is not considered sufficient evidence for activity in this assay. However, responses at two or more treatment levels attaining the 95% confidence level and exhibiting evidence of a dose relationship are considered as evidence for transformation. Responses achieving the 99% confidence level for one or more test material treatments are usually considered sufficient for a positive evaluation.

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